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(71) Applicant: ADVANCED TECHNOLOGIES (CAMBRIDGE) LIMITED [GB/GB]; Globe House, 1 Water Street, London WC2R 3LA (GB).

(72) Inventor: BURRELL, Michael, Meyrick; Advanced Technologies (Cambridge) Limited, 210 Cambridge Science Park, Cambridge CB4 0WA (GB).

(74) Agents: WALFORD, Margot, Ruth et al.; British-American Tobacco (Investments) Limited, Patents Department, R & D Centre, Regents Park Road, Southampton SO15 8TL (GB).

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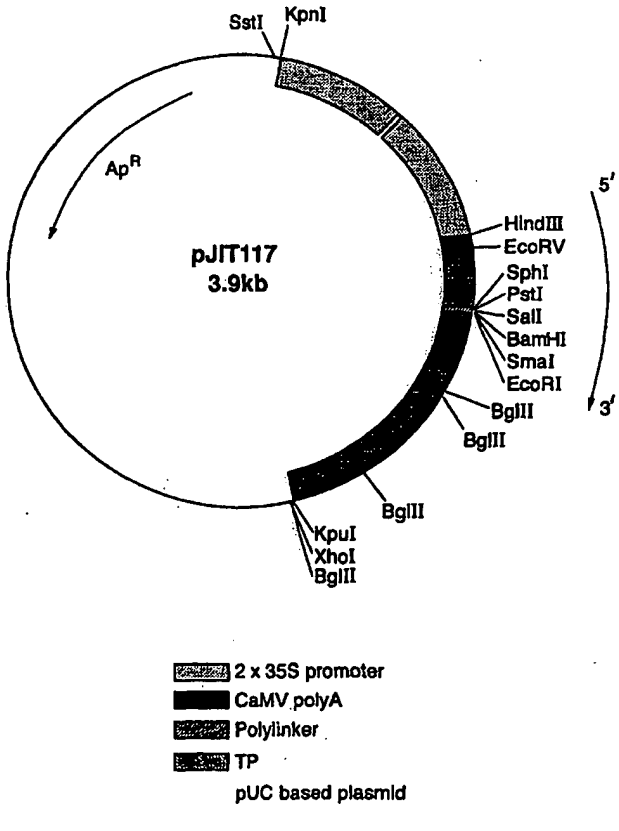
(54) Title: GENETICALLY MODIFIED PLANTS WITH ALTERED STARCH

(57) Abstract

Starch of wheat and maize plants is altered by the introduction of a chimaeric gene comprising a glycogen synthase coding sequence under the control of a promoter directing expression and a terminator. A transit peptide for translocation of the glycogen synthase to the plant plastid may also be included in the chimaeric gene. The starch has altered processing characteristics, in particular an increased chain length.



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Genetically Modified Plants with altered Starch

This invention relates to genetically modified plants, and in particular to genetically modified maize and wheat. The genetically modified plants have an altered starch synthesising ability following the introduction, by recombinant DNA techniques, of one or more gene sequences coding for enzymes in the starch or glycogen biosynthetic pathway into the plant.

Starch is a complex polymer of glucosyl residues. It is the major form in which carbohydrate is stored in the tissues and cells of most species of higher plants. It is accumulated in the leaves of plants during the day as a result of photosynthesis and is used to supply the needs of the plant for energy and biosynthesis during the night. Starch is also accumulated in non-photosynthetic cells, especially those involved in reproduction such as in seeds, fruits and tubers. Therefore, starch is of great importance to the productivity of the plant and its survival.

Starch is also highly significant to man. Firstly, it forms a major component of animal diets, supplying man and his domestic animals with a large portion of their carbohydrate intake. Secondly, the type of starch in a plant affects the quality of the processed plant product. Thirdly, starch is used industrially in the production of paper, textiles, plastics and

adhesives, as well as providing the raw material for some bio-reactors. Starch from different species have preferred uses. On a world scale, starch producing crops are agriculturally and economically by far the most important, and these crops include wheat, maize, rice and potatoes. The type of starch will affect the quality of a processed product and the profitability of the processed crop. In addition, the quantity and quality of starch present in the harvested organ of a plant will affect the gross yield and the processing efficiency.

In plants, i.e. vascular plants, the starch consists of linear chain and branched chain glucans known as amylose and amylopectin respectively. Starch with various amounts of amylose and amylopectin are found in different plants. Typically, plant starch contains 10-25% amylose, the remainder being amylopectin, the branched chain glucan. Amylopectin contains short chains and long chains, the short chains ranging from 5-30 glucose units and the long chains ranging from 30-100 glucose units, or more. It is thought that the ratio of amylose to amylopectin and the distribution of short to long chains in the amylopectin fraction affect the physical properties of starch, e.g. thermal stabilisation, retrogradation and viscosity. These properties also affect the utility of starch, as mentioned above. Starches from different plants have different properties, which also affects their suitability for processing under certain conditions and for certain uses. It

can be seen, therefore, that modifying the starch generated in a plant can have particular utility in the downstream processing or the yield of the starch in the plant storage organ.

Waxy corn starch lacks amylose and this starch has unique properties. Also, most mutations in the waxy locus of maize, which encodes starch granule bound synthase I (GBSSI), result in plants which produce much reduced amylose. When no functioning GBSSI is synthesised in the homozygous waxy mutant it also lacks amylose (Echt & Schwartz, 1981).

The genetic modifications of the present invention produce altered starch composition and properties, which properties are ideally beneficial in terms of starch processing.

In the last few years this concept of modifying starch properties has been postulated and put into practice in varying degrees. In the patent literature International Patent Application, Publication No. WO 94/11520 (Zeneca) described constructs having a target gene which encodes an enzyme involved in the starch or glycogen biosynthetic pathway under control of a gene switch, for example, a chemical or temperature controlled on-off mechanism. Various crops were postulated as being suitable for use in the method but no plant transformation was actually carried out. Some constructs were made but no examples or results were given. International Patent Application, Publication No. WO 94/09144 (Zeneca) was very similar to the just described application. Only the first steps in the

transformation process were demonstrated. No results are given for any plant, and only the transformation of tomato is described with reference to the exemplary methodology, although other plants are mentioned. International Patent Application, Publication No. WO 92/11376 (Amylogene) described introducing antisense genes for GBSSI in to potatoes to down-regulate amylose production with the intention of producing a potato with practically no amylose-type starch. Whilst great detail is given of methodology, no actual results from transformed plants are given and no plant transformations other than potato are postulated. Only a small number of constructs are actually produced to enable one to carry out the invention. The results for potato were eventually published in the scientific literature by Visser *et al* in 1991. Increases in the amylopectin content of the starch was seen. Further scientific papers on altering GBSSI in potato using antisense GBSSI constructs, e.g. Visser *et al* (1991a) and Kuipers *et al* (1994), have shown actual transformation and alteration of starch composition.

In terms of successful transformation using non-plant derived starch-related genes, in International Patent Application, Publication No. WO 92/11382 (Calgene) and their later publication (Shewmaker *et al*, 1994) potato was actually transformed with *E.coli glgA* (Glycogen synthase) and *E.coli glgC* (ADPG pyrophosphorylase). Higher specific gravity measurements

were obtained from transformed potato plants compared with two control events, as well as altered starch characteristics.

It can be seen, therefore, that work to date has involved introducing certain genes involved in glycogen biosynthesis specifically into potato. The effects and their potential usefulness for other plants and other non-plant derived starch-related genes has only been postulated.

This invention seeks to transform cereal crops and specifically wheat and maize with an enzyme involved in the synthesis of microbial glycogen, namely glycogen synthase (E.C. 2.4.1.21).

This invention also seeks to identify properties of the starch in these transformed plants which are particularly useful and/or advantageous in the downstream processing of starch or the plant itself.

The present invention provides transgenic wheat or maize plants, said plants having therein a chimaeric gene comprising a promoter, a coding sequence for glycogen synthase, which coding sequence is derived from a microorganism, and a terminator.

As used herein, the term chimaeric gene refers to a combination of nucleic acid sequences for each part of the chimaeric gene, which sequences have been engineered into relationship by recombinant DNA techniques, which sequences may also be in their separate parts endogenous or exogenous to the plant into which the chimaeric gene is to be introduced.

A construct and a chimaeric gene comprising nucleic acid causing the expression of the sequences above mentioned are also aspects of the invention.

Plant cells containing a chimaeric gene comprising a nucleic acid sequence encoding glycogen synthase are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a chimaeric gene according to the invention.

The present invention also provides a method of altering the starch in maize or wheat plants, the method comprising the steps of stably introducing into the plant genome a nucleic acid sequence encoding glycogen synthase under the direction of a suitable promoter and a suitable terminator, and regenerating a plant having an altered genome.

The present invention also provides a starch obtained from transformed wheat or maize, said starch having an altered chain length and/or processing property compared with control starch from a non-transformed plant.

The chain length and/or branching of the starch may be increased or decreased. Evidence to date suggests that the chain length is increased. This may be associated with decreased branching. Other parameters which may be altered include the degree of retrogradation, the viscosity, the pasting temperature, the gelling temperature, each of which may be

increased or decreased. The starch may also have modified properties for chemical derivitisation.

The turnover of starch in leaves is of central importance to the growth of the plant. A change in the structure of the starch in the granule without a complementary change in other enzymes of starch breakdown might be expected to restrict the export of carbon from the leaf at night. This might be expected to cause an altered ratio of source to sink with a subsequent effect on yield.

Preferably the promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant. The promoter may be heterologous or homologous to the plant. Preferably the promoter directs expression to the endosperm of the plant seed. A preferred promoter is the high molecular weight glutenin (HMWG) gene of wheat. Other suitable promoters will be known to the skilled man, such as the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin, for example.

Preferably the chimaeric gene also contains a sequence that encodes a transit peptide which provides for translocation of the glycogen synthase and/or a marker gene or other coding sequence to the plant plastid. Suitable transit peptides include those from the small sub-unit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) from pea, maize or sunflower, for example. Combinations of transit peptides may

also be used. Other suitable transit peptides for transporting to the amyloplast will be known to those skilled in the art, such as the transit peptide for the plant plastid acyl carrier protein (ACP) or for GBSSI.

The coding sequence encoding glycogen synthase is advantageously a sequence obtained from a microorganism, such as a unicellular organism, algae or bacteria, or alternatively from a mammalian sdource, which sequence has the necessary ability to encode glycogen synthase.

Suitably the glycogen synthase is derived from a bacterial source such as *E.coli* (for example, Baecker, P.A. *et al*, 1983 or Kumar, A. *et al* 1986), *Agrobacterium* (Uttaro, A.D., & Ugalde, R.A. 1994), *Salmonella* (Leung, P.S.C. & Preiss, J. 1987), or *Bacillus* (Kiel, J.A. *et al* 1994). Standard methods of cloning by hybridisation or polymerase chain reaction (PCR) techniques may be used to isolate the sequences from such organisms: for example, molecular cloning techniques such as those described by Sambrook, J. *et al* 1989 and the PCR techniques described by Innis, M.A., *et al* 1990. Other microbial sequences may be obtained in a similar manner.

The chimaeric gene may comprise one or more additional coding sequences from the starch or glycogen biosynthetic pathway, such as, for example, branching enzyme (EC 2.4.1.18).

The transformation techniques for the method of the invention are advantageously direct DNA transfer techniques,

such as electroporation, microinjection or DNA bombardment (the biolistic approach). Alternatively, plant cell transformation using plant vectors introduced into plant pathogenic bacteria, such as *Agrobacterium*-mediated transfer (Cheng, M. *et al* (1997)), may be used. In both methods selectable markers may be used, at least initially, in order to determine whether transformation has actually occurred. Useful selectable markers include enzymes which confer resistance to an antibiotic, such as gentamycin, hygromycin, kanamycin and the like. Alternatively, markers which provide a compound identifiable by a colour change, such as GUS, or luminescence, such as luciferase, may be used.

The chimaeric gene may also comprise a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed. The gene switch may be a chemically induced promoter or a temperature controlled promoter, for example.

In order that the invention may be easily understood and readily carried into effect, reference will now be had, by way of example, to the following diagrammatic drawings in which:

Figure 1 shows a map of the plasmid pJIT117 used in the preparation of the plamid of Figure 2;

Figure 2 shows a map of the plasmid pBS17R used in the sticky-feet polymerase chain reaction;

Figure 3 shows a diagrammatic representation of the steps in the sticky-feet polymerase chain reaction;

Figure 4 shows a map of the plasmid pBSHMWGP used in the preparation of the plasmid of Figure 6;

Figure 5 shows a map of the plasmid pDV02000 used in the preparation of the plasmid of Figure 6;

Figure 6 shows a map of the plasmid pDV03000 used in the preparation of the plasmid of Figure 7;

Figure 7 shows a map of the plasmid pDV03191 according to one aspect of the invention and used in the transformation process of the invention;

Figure 8 shows a standard chromatogram of glucose at 1mM concentration;

Figure 9 shows a standard chromatogram of maltose at 1mM concentration;

Figure 10 shows a standard chromatogram of maltotriose at 1mM concentration;

Figure 11 shows a standard chromatogram of maltohexaose at 1mM concentration;

Figure 12 shows a standard chromatogram of a mixture of maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose each at 1mM concentration;

Figure 13 shows a chromatogram of an isoamylase digest of wheat starch from wheat plants according to the invention;

Figure 14 shows a graph of starch branch chain lengths for starch from the seed of a single transgenic wheat plant compared with starch from the seed of a control wheat plant;

Figure 15 shows a graph of starch branch chain lengths for starch from the seed of a further single transgenic wheat plant compared with starch from the seed from a control wheat plant;

Figure 16 shows a comparison of branch chain length for a family of starches from the seed of transgenic lines against a family of starches from the seed of control wheat plants;

Figure 17 shows a western blot of proteins extracted from the seed of transgenic maize plants according to the invention. 1 (A-F) refers to the proteins extracted from different seeds of the maize plant 2 AM4-5' - 2; and 2 (A-B) refers to proteins extracted from the seeds of the maize plant 2-AM4-6'-1. -ve is a non transgenic control.

The invention will now be described, by way of example, with reference to an embodiment for incorporating *glgA* from *E.coli* strain LCB618 into wheat and maize.

EXAMPLE 1

Construction of *glgA* plasmids used for particle bombardment of wheat embryos.

Isolation of *E.coli* chromosomal DNA

The coding sequence for *glgA* was originally isolated by PCR using chromosomal DNA from the *E.coli* strain LCB618 as

template. *E.coli* LCB618 was obtained from *E.coli* Genetic Stock Center, Yale University, U.S.A.

E.coli LCB618 was grown up in 100ml LB o/n at 37°C. Cells were pelleted and re-suspended in 9.5ml 10mM Tris-HCl, 1mM EDTA (TE) pH8.0 and 0.5ml 10% w/v Sodium dodecyl sulphate (SDS) and 50μl proteinase K 20mg/ml were added. The mixture was incubated at 37°C for 1 hour to lyse cells. 1.8ml of 5M NaCl followed by 1.5ml of CTAB (cetyl trimethyl ammonium bromide)/NaCl solution (10% w/v CTAB in 0.7M NaCl) were added and the mixture incubated at 65°C for 20 minutes. The lysate was extracted with an equal volume of chloroform and centrifuged at 6000g to separate the layers. The upper layer was removed to a fresh tube and DNA was precipitated by the addition of 0.6 volumes isopropanol. The DNA was removed from the solution with a sealed pasteur pipette, placed into a fresh tube and washed with 70% ethanol. The DNA was dried *in vacuo* and re-suspended in TE pH8.0. The DNA was purified on a CsCl gradient.

Sticky-feet PCR

In order for the *E.coli* glycogen synthase to function in plants the protein has to be transported into the amyloplast. This transport can be facilitated by attachment of a plastid transit peptide to the amino terminus of the *E.coli* polypeptide.

The coding sequence for the transit peptide (TP) from the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) pea has been cloned and the TP shown to target β-glucuronidase (GUS) protein to chloroplasts (Guerineau *et al*, 1988).

The plasmid pJIT117 (Guerineau *et al*, 1988), the map of which is shown in Figure 1, has several restriction sites downstream of the ssuTP which can be used for subcloning of coding sequences, however, the subcloning must create a translational fusion between the transit peptide and the coding sequence, and the Cys-Met amino acid sequence at the junction must be maintained.

We have previously used pJIT117 to attach the ssu transit peptide to the coding sequence for *E.coli* ADPG PPase *glgC16* using restriction digestion and PCR. The TP-*glgC16* DNA, herein known as SEQ.ID. No.1, was subsequently transferred to the vector pBluescript (Stratagene Ltd., Cambridge, UK) to create pBS17R (the map for which is shown in Figure 2) and this plasmid was useful in generating a similar construct for *glgA*.

The *glgA* coding sequence has no convenient restriction sites at the 5' end. Therefore, to ensure that the open reading frame was in a translational fusion with the ssu transit peptide and to maintain the integrity of the Cys-Met cleavage site, plasmid pBS17R was used to substitute the *glgA* sequence for the

glgC16 sequence with a technique called sticky-feet PCR (Clackson and Winter, 1989).

This technique is explained diagrammatically with reference to Figure 3. In this technique, PCR primers are designed to the 5' and 3' ends of the acceptor sequence of chromosomal or genomic DNA and the sequences which are to be attached to the acceptor from a donator plasmid. In Step A, PCR is used to amplify the sequences which are to be inserted in the donator. In Step B the amplified acceptor DNA fragment is annealed to the donator plasmid which has been made single-stranded and carries uracil residues instead of thymidine residues by using a specific type of *E.coli* host. In Step C, a new strand is synthesised, using the donator plasmid as template and the acceptor fragment as primer, with a combination of *Taq* polymerase, T7 DNA polymerase (Sequenase) and T4 DNA ligase. The new double-stranded plasmid is a hybrid with one strand of the uracil-containing donator and one strand incorporating the acceptor fragment.

This hybrid plasmid is then transferred into a normal *E.coli* host where the uracil-containing strand is degraded and the acceptor strand replicated. A double-stranded plasmid incorporating the acceptor DNA can then be recovered. As an alternative, in Step D (not shown), the hybrid plasmid can be used in a PCR reaction with primers which will amplify out the

acceptor DNA with the required fragments from the donator attached.

In this particular example, *glgA* sticky-feet primers were designed as follows:

SEQ. ID. No. 3 GLGASF5 (P1)

TGGTGGAAGAGTAAAGTGCATGCAGGTTTTACATGTATGTTCA

← ssu TP 3'end | *glgA* 5' end →

SEQ. ID. No. 4 GLGASF3 (P2)

TCGCTCCTGTTTATGCCCTAGATCTCTATTTCGAGCGATAGTAAAGCTCACGGT

←*glgC* 3'end | *glgA* 3' end→

The PCR primers are designed to the 5' and 3' ends of the *glgA* cDNA sequence.

The 5' end primer (SEQ. ID. No. 3) also has sequences which are homologous to the ssu-TP.

The 3' end primer (SEQ. ID. No. 4) also incorporates sequences which are homologous to the 3' end of the *glgC* coding sequence. These primers are used in a PCR process to amplify a *glgA* fragment with extensions which will overlap onto the sequences in pBS17R. This is represented by Step A of Figure 3.

Plasmid pBS17R is made into a template for sticky-feet PCR by transferring the plasmid into the *E.coli* host CJ236 (Raleigh *et al*, 1989). This host is deficient in the enzyme dUTPase, (i.e. dut$^-$) which results in deoxyuridine being incorporated into the DNA instead of thymidine. The absence of another enzyme

uracyl N-glycosylase (ung$^-$) means that the deoxyuridines cannot then be removed from the DNA.

In Step B of Figure 3, the extended *glgA* DNA (2) is annealed to the uracil-containing template which has been isolated as single-stranded DNA (3), and a new strand is synthesised as per Step C above. The new double-stranded plasmid is a hybrid (5) with one strand of the uracil-containing template (3) and the other strand consisting of the plasmid backbone and the *glgA* fragment now with ssu-TP and a 3' *glgC* fragment attached at 5' and 3' ends respectively (4).

In Step D (not shown), the hybrid plasmid is used in a PCR reaction with primers (SEQ. ID. No. 5) (P3)(see below) and SEQ. ID. No. 4 (P2) which will amplify out the extended *glgA*.

With reference to Figure 3, the experimental details are as follows:

The primers GLGASF5 (P1) (SEQ. ID. No. 3) vs GLGASF3 (P2) (SEQ. ID. No. 4) were kinased and used to amplify the *glgA* open reading frame with extension sequences using *E.coli* LCB618 genomic DNA (1) as template. The DNA (2) was purified with GeneClean (BIO 101, Ltd.). The sticky-feet template DNA, single-stranded uracil pBS17R DNA (3), was isolated from 5ml overnight cultures of the *dut$^-$ ung$^-$ E.coli* strain CJ236.

The sticky-feet PCR reaction was carried out in 10μl volume containing 20ng ss uracil pBS17R (3); 200ng *glgA* DNA (2), 1μl x 10 Taq polymerase buffer, 1.0μl 2mM mixture of dATP, dTTP, dCTP,

dGTP (2mM dNTPs); 2.5 units Taq polymerase. The mix was overlaid with 30μl mineral oil and cycled once at 94°C, 3 min; 72°C, 2 min; 40°C, 2 min. and then cooled to room temperature. 10μl of a solution containing 2.0μl x5 Sequenase buffer (200mM Tris-HCl pH 7.5; 100mM $MgCl_2$, 250mM NaCl), 1.5μl 0.1mM Dithiothreitol (DTT); 2.0μl 10mM Adenosine 5' triphosphate (ATP); 4 units T4 DNA ligase; 6.5 units Sequenase was then added and the reaction incubated at room temperature for 30 minutes.

Generation of TP-glgA DNA

1.0μl of the reaction containing the hybrid plasmid (3 + 4) was taken and diluted to 10μl with 10mM TE at pH8.0. 1.0μl of the diluted sample was used in a PCR reaction in order to obtain the TP-*glgA* coding sequence (Step C of Figure 3). The primers used were TPSSU5 (P3) (SEQ. ID. No. 5) vs GLGASF3 (P2) (SEQ. ID. No. 4).

SEQ. ID. No. 5 TPSSU5 (P3)

ACGTAGATCTATGGCTTCTATGATATCCTCTTC

The primers both have restriction sites for *BglII*, therefore after purification, the amplified DNA was digested with *BglII* and subcloned into the *BamHI* site of pDV03000 (see below).

Construction of pDV03000 vector

Transgenic wheat and maize plants are generated by particle bombardment of embryos and it is not necessary to use binary vectors. For expression of the *glgA* protein the coding sequence is placed under the control of an endosperm-specific promoter. One such suitable promoter is that from the High Molecular Weight Glutenin (HMWG) gene of wheat (Bartels and Thompson, 1986). Primers (*P4*) and (*P5*) (SEQ. ID. Nos. 6 and 7 respectively) were designed so that the 430bp HMWG promoter, (the nucleotide sequence of which is given in SEQ. ID. No. 2) could be isolated by PCR and subcloned via *EcoRI* and *ClaI* restriction sites into pBluescript to generate the plasmid pBSHMWGP (Figure 4).

A second set of PCR primers were designed to obtain the nopaline synthase terminator from plasmid pDV02000, the map of which is shown in Figure 5. This plasmid was previously constructed in our laboratory as an intermediate vector for the sub-cloning of coding sequences. The 5' primer, NTPRIME5 (*P6*) (SEQ. ID. No. 8), has a *BamHI* restriction site, while the 3' primer NTP3NXS2 (*P7*) (SEQ. ID. No. 9), has restriction sites for *NotI, XhoI* and *SacII*. The amplified DNA was digested with *BamHI* and *SacII* and ligated into the pBSHMWGP plasmid to generate pDV03000 (the map of which is shown in Figure 6).

SEQ. ID. No. 6 HMWGPRO5 (*P4*)

GACATCGATCCCAGCTTTGAGTGGCCGTAGATTTGC

SEQ. ID. No. 7 HMWGPRO3 (*P5*)

GACGAATTCGGATCTCTAGTTTGTGGTGCTCGGTGTTGT

SEQ. ID. No. 8 NTPRIME5 I(*P6*)

CAGGATCCGAATTTCACCCGATCGTTCAAACA

SEQ. ID. No. 9 NTP3NXS2 (*P7*)

GACCCGCGGCTCGAGGCGGCCGCCCGATCTAGTAACATAGATGACACCGC

pDV03000 vector has the HMWG promoter-nos terminator sequences separated by unique restriction sites for *EcoRI*, *PstI*, *SmaI* and *BamHI*.

Construction of pDV03191

TP-*glgA* DNA amplified from the sticky-feet PCR sample with primers TPSSU5 vs GLGASF3 (Step D, Figure 3) was digested with *BglII*, purified and ligated into the *BamHI* site of pDV03000. Plasmid pDV03191 (the map of which is shown in Figure 7) was confirmed by restriction enzyme digestion and by sequencing of the junctions between promoter and coding sequence. *E.coli* XL1 Blue (Stratagene Ltd., UK) harbouring pDV03191 was deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge

Science Park, Cambridge CB4 0WA, under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collection of Industrial and Marine Bacteria (NCIBM) 23 St. Machar Street, Aberdeen, Scotland, GB on 4 August 1998 under accession number NCIMB 40962. The micro-organism is *E.coli* XL1 Blue : strain LCB618 containing pDV03191. The DNA for *E.coli glgA* was inserted as described above into pBluescript with the ssu transit peptide, the HMWG promoter and nos terminator. The vector is useful for altering starch properties.

Transformation of wheat

Methods for the transformation of wheat by particle bombardment are well known in the art, for example see Vasil *et al*, 1992.

Immature embryos of wheat are used to initiate embryogenic callus. The callus is subcultured and used for particle bombardment with gold particles coated with plasmid DNA.

Two plasmids are used per bombardment, one plasmid carries the construct of interest, in this case pDV03191. The second plasmid carries the selectable marker which expresses the gene responsible for resistance to the herbicide Basta. Plants resistant to Basta are generally found to also have the recombinant gene of interest present.

Bombarded calli are grown on Basta selection media and surviving calli are transferred to regeneration medium. Rooted plants are transferred to soil and grown to maturity in a growth room.

Primary transformant wheat plants (T_0) are selfed to produce transgenic seed.

Seed are extracted for protein and the protein analysed by western blotting for the presence of *E.coli glgA* polypeptide.

EXAMPLE 2

Biochemical Analysis of *glgA* transformed maize

1. Expression of *glgA* protein

Soluble protein samples were prepared from individual maize grain derived from transformed maize plants. Each grain was pulverised in a pestle and mortar until a fine powder was obtained. A portion of this powder (100-200mg) was placed in an Eppendorf tube and 500µl of ice cold extraction buffer (50mM HEPES, pH 8.0; 10mM DTT; 10mM EDTA) added. The powder was homogenised with a micropestle to release soluble proteins.

The extract was centrifuged at 13000 rpm for 1 minute and the supernatant decanted into a fresh Eppendorf tube and stored on ice. The total protein content in the soluble protein sample was assayed using The Bradford dye binding method (Bradford, M. 1976).

An aliquot of the soluble protein sample containing 100mg total protein was placed into an Eppendorf tube and excess acetone (ca 1.5ml) was added to precipitate the proteins. The proteins were collected by centrifuging the sample at 13000 rpm for 5 minutes. The acetone was decanted off and the samples were air-dried until all the residual acetone had evaporated.

SDS PAGE loading buffer (4% (w/v) SDS; 12% (w/v) glycerol; 50mM Tris-HCl pH 6.8; 2% (v/v) β-mercaptoethanol; 0.01% Serva blue G) in an amount of 100μl was added to the protein sample contained in the Eppendorf tube. Samples were boiled for 1 minute before loading onto a polyacrylamide gel. Electrophoresis was carried out according to the method of Schägger and Von Jagow (1987). The resolving gel composition was 10% acrylamide, 3% bis-acrylamide. Gels were run at 50 V constant for 16 hours.

Separated proteins were transferred from the acrylamide gel onto PVDF membrane by electroblotting (Transfer buffer: 20% methanol; 25mM Tris-HCl pH 8.3; 190mM glycine. Run in a Biorad blotting apparatus at 50 V for 3 hours).

To detect expression of *glgA* the membrane was challenged with a rabbit anti-*glgA* antiserum (raised *glgA*-GST fusion protein purified from *E.coli*). Specific cross-reacting proteins were detected using an anti-rabbit IgG-alkaline phosphatase conjugate secondary antibody and visualised by the NBT/BCIP reaction.

NuPage™ Electrophoresis

Alternatively, an aliquot of the soluble protein sample, containing 100mg total protein was placed into an Eppendorf tube and excess acetone (ca 1.5ml) was added to precipitate the proteins. The proteins were collected by centrifuging the sample at 13000 rpm for 5 minutes. The acetone was decanted off and the samples were air-dried until all the residual acetone had evaporated.

NuPage™ loading buffer (2% (w/v) SDS; 10% (w/v) sucrose; 25 mM Tris-HCl pH 8.5; 1% (v/v) β-mercaptoethanol; 0.5 mM EDTA; 0.02% Serva blue G250; 0.006% Phenol Red) 100 μl, was added to the protein sample contained in the Eppendorf tube. Samples were heated at 100°C for 1 minute before loading onto a polyacrylamide gel. Electrophoresis was carried out on NuPage™ precast gels according to the manufacturer's instructions (Novex, San Diego CA). Gels were run at 200 V constant for 60 minutes using MES SDS running buffer (20 mM MES/20 mM Tris-HCl pH 7.3; 1% (w/v) SDS; 1 mM EDTA).

Separated proteins were transferred from the acrylamide gel onto PVDF membrane by electroblotting (Transfer buffer: 20% methanol; 25 mM Bis-Tris/25 mM Bicine pH 8.3; 1 mM EDTA. Run in a Novex electroblotting apparatus at 25 V for 1.5 hours).

To detect expression of *glgA* the membrane was challenged with a rabbit anti-*glgA* antiserum (raised against *glgA*-GST fusion protein purified from *E. coli*). Specific cross-reacting

proteins were detected using an anti-rabbit IgG-horse Radish Peroxidase conjugate secondary antibody and visualised using enhanced chemiluminesence (ECL) as supplied by Amersham International.

Several transformed lines were found to express a 50 kDa protein in their grain, which was not present in control grain derived from non-transformed maize plants.

2. Preparation of wheat starch

Starch was extracted from grain of separate field grown samples of two of the *glgA* expressing lines and a control line. Wheat grains of each sample (3-4g) were placed in a mortar, 30ml of 1% sodium bisulphite was added and placed on ice for 30 minutes. The grains were then gently pulverised using a pestle. The solution was filtered through a nylon filter sieve and collected in a centrifuge tube. The pulverised wheat grains were re-extracted with a further 30ml of 1% sodium bisulphite and the filtrates were combined. The filtrate was centrifuged at 6000 rpm for 5 minutes. After decanting off the supernatant, the pellet of extracted starch was re-suspended in water and centrifuged at 6000 rpm for 5 minutes. This was repeated once. The resulting starch pellet was re-suspended in acetone, centrifuged at 6000 rpm for 5 minutes and the supernatant decanted away. This was repeated once and the starch left to air dry. Once dried the starch was stored at -20°C.

3. **Branch chain length analysis of wheat starch**

Portions of the starch samples were digested with isoamylase and the resulting unbranched linear glucan chains were analysed by HPLC.

75mg of isolated wheat starch was placed in a 15ml Pyrex boiling tube and suspended in 3.0ml of water. The sample was placed in a boiling water bath for 6 minutes, occasionally removed and vortex mixed. The sample was cooled to room temperature and 250μl of 200mM sodium acetate, pH 3.5 and 180 units of isoamylase enzyme added. The samples were made up to a final volume of 3.8ml with water. After mixing, the sample was placed in a 37°C water bath for 4 hours. The samples were occasionally vortex mixed throughout this incubation period. At the end of the incubation the sample was placed in a boiling water bath for 2 minutes, and then allowed to cool to 4°C. The sample was centrifuged at 3,400 rpm for 20 minutes. The resulting supernatant was transferred to Eppendorf tubes and centrifuged at 13000 rpm for 15 minutes. Finally, the sample was filtered through a 0.2mm syringe filter and stored at 4°C until required.

Separate isoamylase digest samples were normalised to a constant total glucan content by digesting a portion of the sample to glucose using α-amylase and amyloglucosidase.

Two 100μl aliquots of isoamylase digested starch were placed in two separate Eppendorf tubes (one is to be used as a blank). To one aliquot was added: 500μl of 200mM sodium acetate pH 4.8; 50μl of α-amylase solution containing 10 units of α-amylase; 100μl of amyloglucosidase solution containing 10 units of amyloglucosidase and water to a final volume of 1.0ml. To the second (blank) aliquot was added: 500μl of 200mM sodium acetate pH 4.8 and 400μl of water. The samples were left to digest at 25°C for 16 hours.

The glucose content of the digest and blanks was assayed spectrophotometrically using a coupled enzyme assay. An aliquot of the total glucose digest or the blank was added to a cuvette containing in a final volume of 990 μl 100mM HEPES, pH 8.0; 5mM $MgCl_2$; 4mM NAD; 1mM ATP and 1 unit of hexokinase. The optical density (OD) of the reaction mixture at 340nm was measured prior to the addition of 10μl containing 1 unit of glucose-6-phosphate dehydrogenase. The OD at 340nm was monitored until there was no further change and the difference in OD after the addition of glucose-6-phosphate dehydrogenase compared to before the addition of glucose-6-phosphate dehydrogenase was determined. This figure was used to determine the total glucose amounts in the original isoamylase digests. These samples were diluted with water to a standard concentration of 8mM total glucose and stored at 4°C until required for HPLC analysis.

The samples were then analysed by Dionex HPLC using a Dionex PA 100 column and PED-Integrated Amperometric detection. The solvent flow rate was 1.0ml/min and a gradient system was developed. Solvent 1 consisted of 100mM NaOH and Solvent 2 was 100mM NaOH, 0.60M sodium acetate. The gradient profile was as shown in Table 1, with the pulsed electrochemical detection (PED) parameters shown in Tables 2.1 and 2.2.

Table 1

Gradient Profile

Event Start Time (min)	Solvent 1 (%)	Solvent 2 (%)
0	100	0
1	100	0
2	100	0
30	0	100
30.1	100	0
35	100	0

Table 2.1

Waveform Table

Time (sec)	Potential (V)
0	0.1
0.5	0.1
0.51	0.6
0.59	0.6
0.6	-0.6

0.65	-0.6

Table 2.2

Integration

Begin (sec)	End (sec)
0.3	0.5

Three isoamylase digestions were performed for each sample and three aliquots of each isoamylase digest were analysed by the HPLC system. Separate chromatogram peaks were assigned to specific linear glucan sizes by reference to standard mixtures containing linear glucans of known numbers of glucose molecules (see Figures 8-12). Peak areas were abstracted from the primary data and averaged for the replicate chromatograms.

Figures 8 to 12 are HPLC traces of standards for various sugars. The standards in Figures 8-12 allow the peak area for each peak of the inventive sample of Figure 13 to be converted to a quantitative representation of the number of glucan chains in each peak, and the position (on the x-axis) of each peak to the number of glucose residues in each chain, i.e. the chain length. In Figures 14 and 15 this conversion has been done for wheat starch extracted from a single transgenic line and its paired control. In Figure 16, a family of starches from transgenic lines are compared with a family of controls. Figure 16 clearly shows that the transgenic starches have a different chain length distribution from the control starches. The starch

in the transgenic seed has a lower percentage of polysaccharide in the chain lengths between 6 and 15 glucose units and an increase in the percentage polysaccharide in the chain length between 17 and 28 glucose units. There is thus an average increase in chain length in the population. The starch has therefore been altered, which alteration affects its processing capabilities.

EXAMPLE 3

Maize plants transformed with *glgA* recombinant gene

In the transformation step, immature maize embryos are used instead of wheat and are subject to particle bombardment with gold particles coated with plasmid DNA. Methods for the transformation of maize are well known in the art, for example see Gordon-Kamm *et al* (1990) and Fromm *et al* (1990). After rooted primary transformant plants (T_0) are transferred to soil and grown to maturity, maize plants are back-crossed to produce transgenic seed which can be extracted and analysed according to Example 2. Further back-crossing is performed to introgres the transgene into elite varieties and selfing of transgenic plants is performed to obtain plants and seed which are homozygous for the transgene. Seed from these generations can also be extracted and analysed according to method 2.

Seed from a number of back-crossed primary transformants were shown to be expressing the *glgA* protein. The plants grown

up from the remaining seeds were subsequently selfed and progeny seed were extracted for protein and western blotting according to Example 2. Figure 17 shows the presence of *glgA* polypeptide in seed from two of these second generation lines 2-AM4-5'-2 and 2-AM4-6'-1.

EXAMPLE 4

Viscometry measurements of transgenic wheat seed extracts.

Flour was extracted from T2 and T3 progeny seed of primary transformant wheat line 72.11B which was shown to be expressing the *glgA* polypeptide by western blots. 4g of ground sample (14% moisture) was mixed with 25ml water or with 24.5ml water + 0.5ml 10% $AgNO_3$ solution. The presence of silver nitrate will inhibit any amylase activity in the slurry and allows the true viscosity developed by the flour to be assessed.

The slurry was subject to rapid viscometric analysis (RVA) using standard profile 1 (Table 3). Results of the RVA are tabulated in Table 4 and Table 5 below.

Standard 1: Idle temperature : 50 ± 1°C

End Test (HH:MM:SS) : 00:13:00

Table 3

Time (HH:MM:SS)	Type	Value
00:00:00	Speed	960 rpm
00:01:00	Speed	160 rpm
00:01:00	Temp.	50°C

00:04:45	Temp.	95°C
00:07:15	Temp.	95°C
00:11:00	Temp.	50°C

Table 4

RVA STD without $AgNO_3$

	Pasting temperature	Peak viscosity	BKD	Final viscosity
CYMMIT control	87.2	191	61	222
72.11B/62	87.2	181	57	208
72.11B/39/4	88.1	182	53	223
72.11B/49/11	86.3	184	53	230
72.11B/41/22	88.1	185	52	226

Table 5

RVA Modified with $AgNO_3$

	Pasting temperature	Peak viscosity	BKD	Final viscosity	Peak $AgNO_3$ - Peak standard	Final viscosity $AgNO_3$ - FV std
CYMMIT ctrl	86.4	251	98	267	60	45
72.11B/62	87.2	251	99	259	70	51
72.11B/39/4	87.3	238	86	265	56	42
72.11B/49/11	87.2	234	80	267	50	37
72.11B/41/22	86.5	244	87	273	59	47

There is thus a fall in the peak viscosity and final viscosity of flour from the transgenic seeds, even after the influence of amylase has been taken into account.

EXAMPLE 5

Differential scanning calorimetry of *glgA* transgenic wheat seed extracts

Wheat kernels were cleaned and water was added to the sample (90mg). The sample was allowed to condition in the analysis chamber at ambient temperature for 24 hours before cycling using the following conditions:

Stabilisation: 1h 25min at 25°C

Raise temperature to 110°C at 1.2°C/minute

Cool to 25°C at 1.2°C/minute.

The DSC results are shown in Table 6.

Table 6

	Peak 1 (amylopectin)			Peak 2 (amylose-lipid complex)		
	Onset Point	Temp. peak	Enthalpy	Onset Point	Temp. Peak	Enthalpy
CYMMIT ctrl.	52	60	6.9	80.2	92.5	1.9
72.11B/62	52	59	6.7	82	93	1.4
72.11B/39/4	52	60	6.8	80	93	1.9
72.11B/49/11	52.3	59.6	6.4	80	93	1.8
72.11B/41/22	51.7	59.4	6.8	80.2	92	1.8

There is a slight increase in enthalpy values for the transgenic seed extracts.

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Materials Abbreviations

LB - Luria broth

TF - Tris-HCl, 1mM EDTA

SDS - sodium dodecyl sulphate

CTAB - cetyl trimethyl ammonium bromide

dATP - 2' - deoxy adenosine 5' triphosphate

dTTP - 2' - deoxy thymidine 5' triphosphate

dCTP - 2' - deoxy cytosine 5' triphosphate

dGTP - 2' - deoxy guanosine

DTT - dithiothreitol

ATP - adenosine 5' triphosphate

HEPES N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

NBT - nitroblue tetrazolium

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

GST - glutathione S transferase

NAD - nicotinamide adenine dinucleotide

IgG - immunoglobulin G

CLAIMS

1. A method of altering the starch in maize or wheat plants, the method comprising the steps of stably introducing into the plant genome a chimaeric gene comprising a nucleic acid sequence encoding glycogen synthase under the direction of a suitable promoter and a suitable terminator, and regenerating a plant having an altered genome.
2. A method according to Claim 1, wherein said nucleic acid sequence encoding glycogen synthase is a sequence obtained from a unicellular organism, an alga or bacterium, which sequence has the necessary ability to encode glycogen synthase.
3. A method according to Claim 1 or 2, wherein said glycogen synthase is derived from *E.coli*, *Agrobacterium*, *Salmonella* or *Bacillus*.
4. A method according to Claim 1, 2 or 3, wherein said promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant.
5. A method according to any one of Claims 1 to 4, wherein said promoter is heterologous or homologous with respect to said plant.
6. A method according to Claims 1, 2, 3, 4 or 5, wherein said promoter directs expression to the endosperm of the seed.
7. A method according to Claim 6, wherein said promoter is the high molecular weight glutenin (HMWG) gene of wheat.

8. A method according to Claim 4, wherein said promoter is one or more of the group consisting of the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin.

9. A method according to any one of Claims 1 to 8, wherein said chimaeric gene also contains a sequence that encodes a transit peptide which provides for translocation of the glycogen synthase and/or a marker gene or other coding sequence to the plant plastid.

10. A method according to Claim 9, wherein said transit peptide is one or more of the group consisting of the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) from pea, maize or sunflower, the transit peptide for the plant plastid acyl carrier protein (ACP) or the transit peptide for GBSSI.

11. A method according to any one of the preceding claims, wherein said chimaeric gene comprises one or more additional coding sequences from the starch or glycogen biosynthetic pathway.

12. A method according to Claim 11, wherein said additional coding sequence is the sequence glycogen branching enzyme (EC 2.4.1.18).

13. A method according to any one of the preceding claims, wherein said chimaeric gene also comprises a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed.

14. A method according to Claim 13, wherein said gene switch is a chemically induced promoter or a temperature controlled promoter.

15. Starch obtained from wheat or maize transformed according to Claims 1-14, said starch having an altered chain length and/or processing property compared with control starch from a non-transformed plant.

16. Starch according to Claim 15, wherein said chain length is increased.

17. Starch according to Claim 16, wherein there is an increase in the percentage polysaccharide in the chain lengths between 17 and 38 glucose units.

18. Starch according to Claim 15, wherein the viscosity is decreased, said altered viscosity affecting the processing properties of said starch.

19. Starch according to Claim 15, wherein the degree of retrogradation of said starch is altered, said altered degree of retrogradation affecting the processing properties of said starch.

20. Starch according to Claim 15, wherein the freeze-thaw stability of said starch is improved.

21. Maize or wheat plant cells containing a chimaeric gene comprising a promoter, a coding sequence for glycogen synthase, and a terminator.

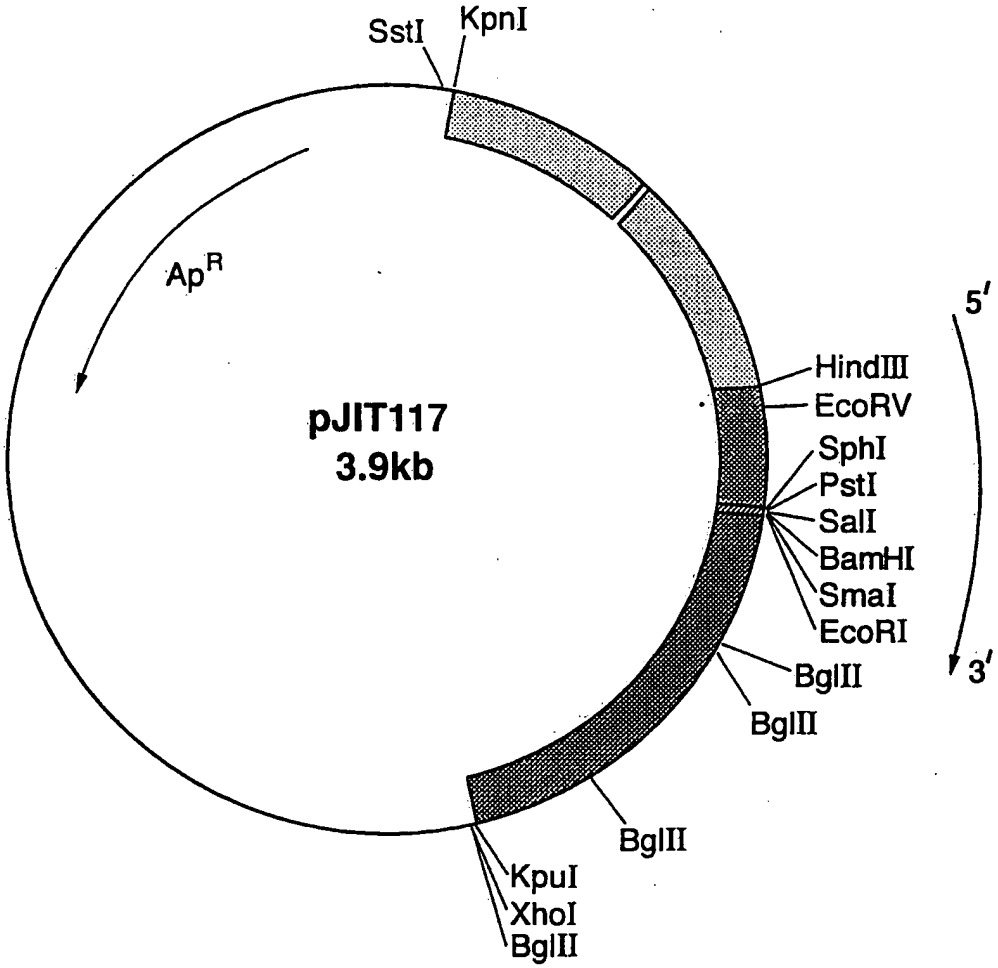
22. Seed of a maize or wheat plant transformed in accordance with any one of Claims 1-14.

23. Maize or wheat plants or cells transformed according to any one of Claims 1-14 and containing starch having a decreased chain length.

24. A construct as described in Figure 7 and deposited under NCIMB Accession No. 40962.

25. A construct comprising a promoter-gene fragment-terminator cassette comprising a transit peptide and coding sequence for glycogen synthase derived from a micro-organism.

Fig.1.



2 x 35S promoter
CaMV polyA
Polylinker
TP

pUC based plasmid

Fig.2.

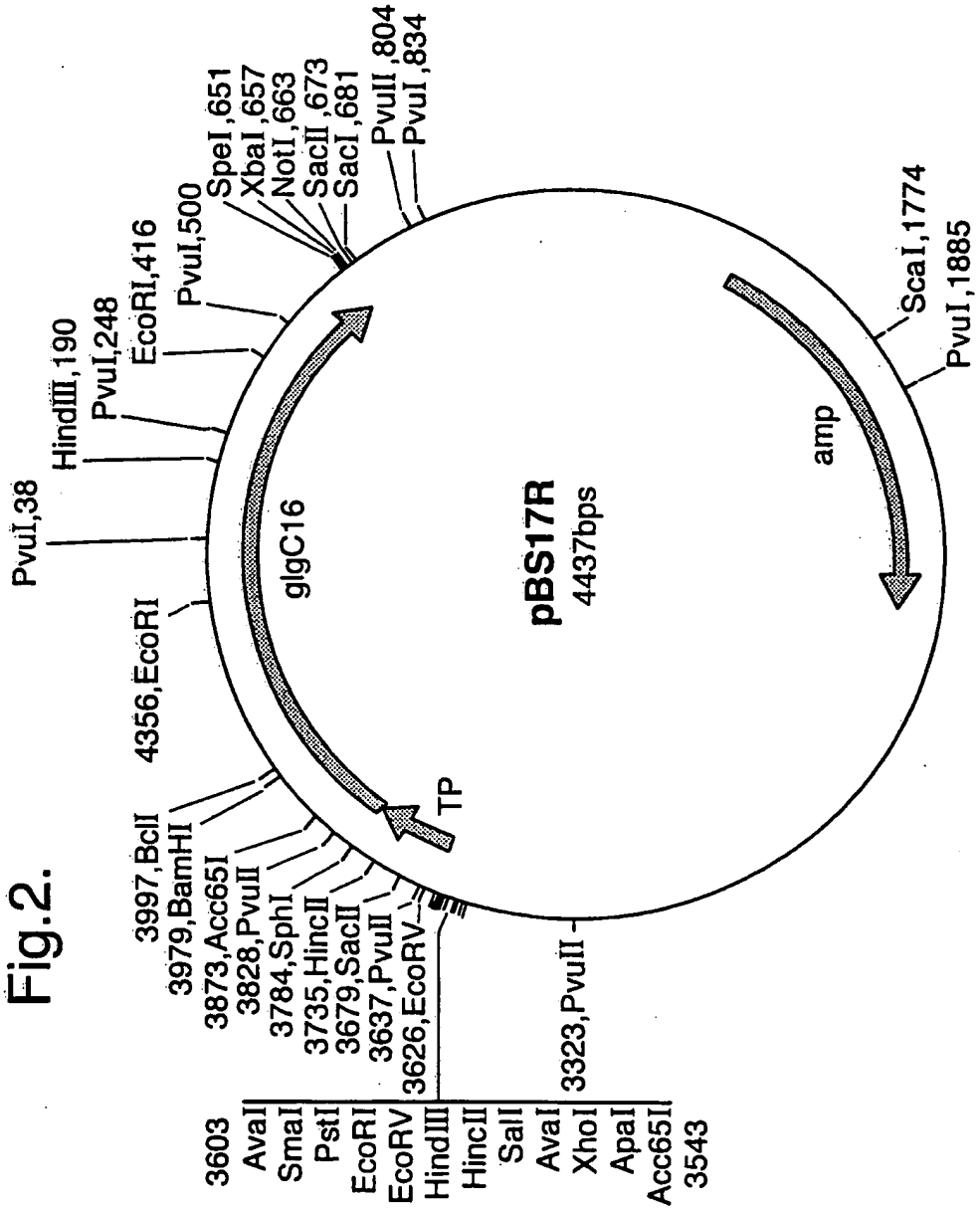

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Fig.3.

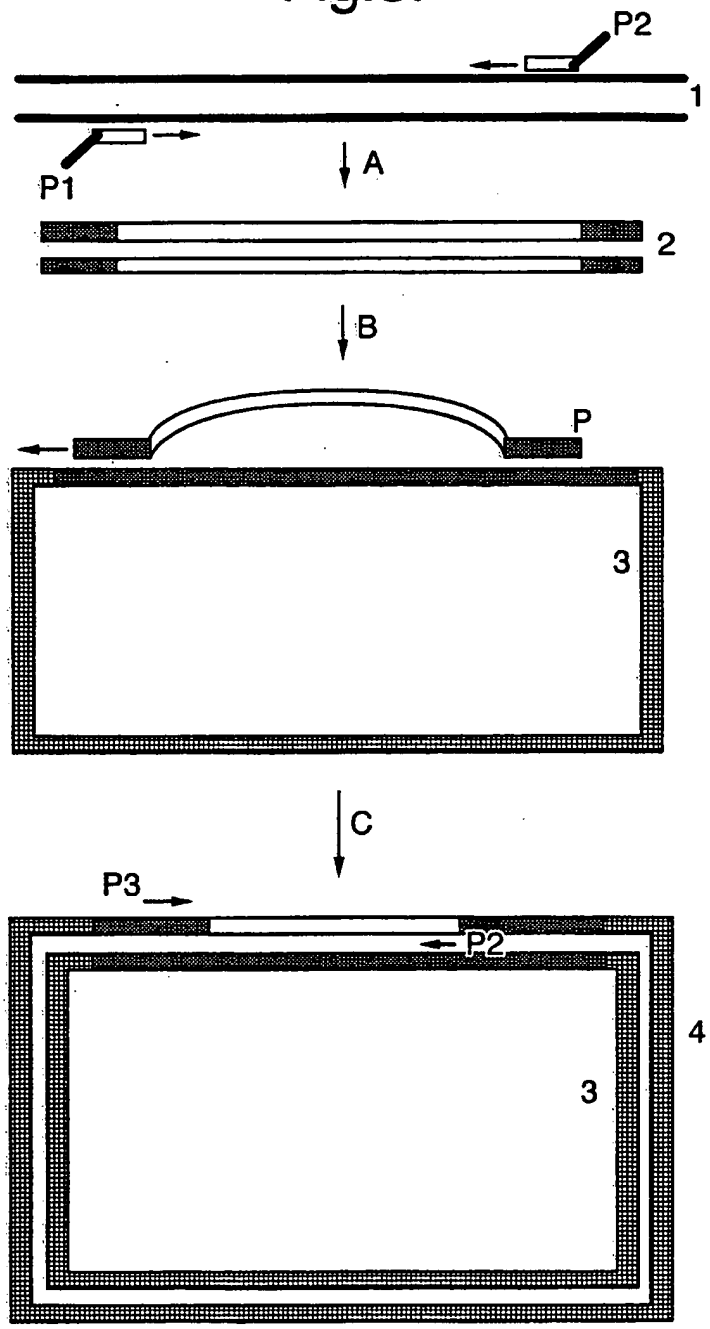

Fig.4.

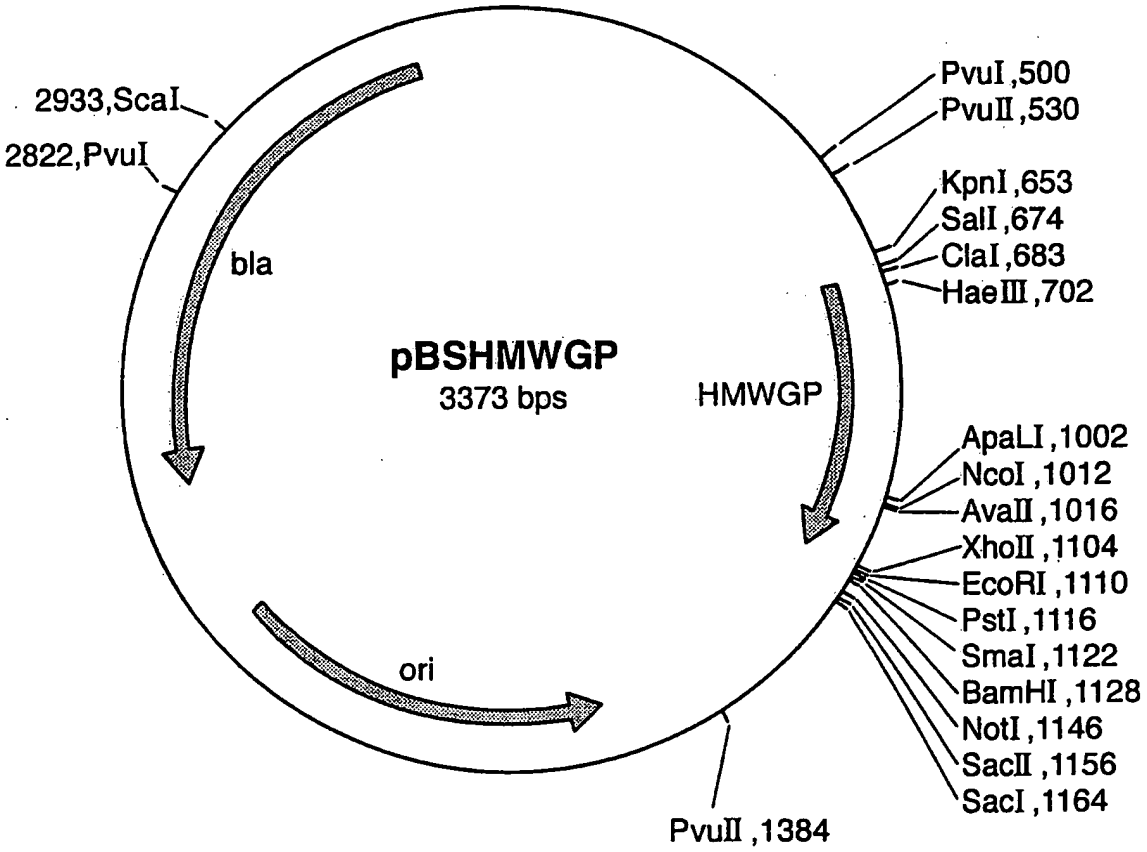
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Fig.5.

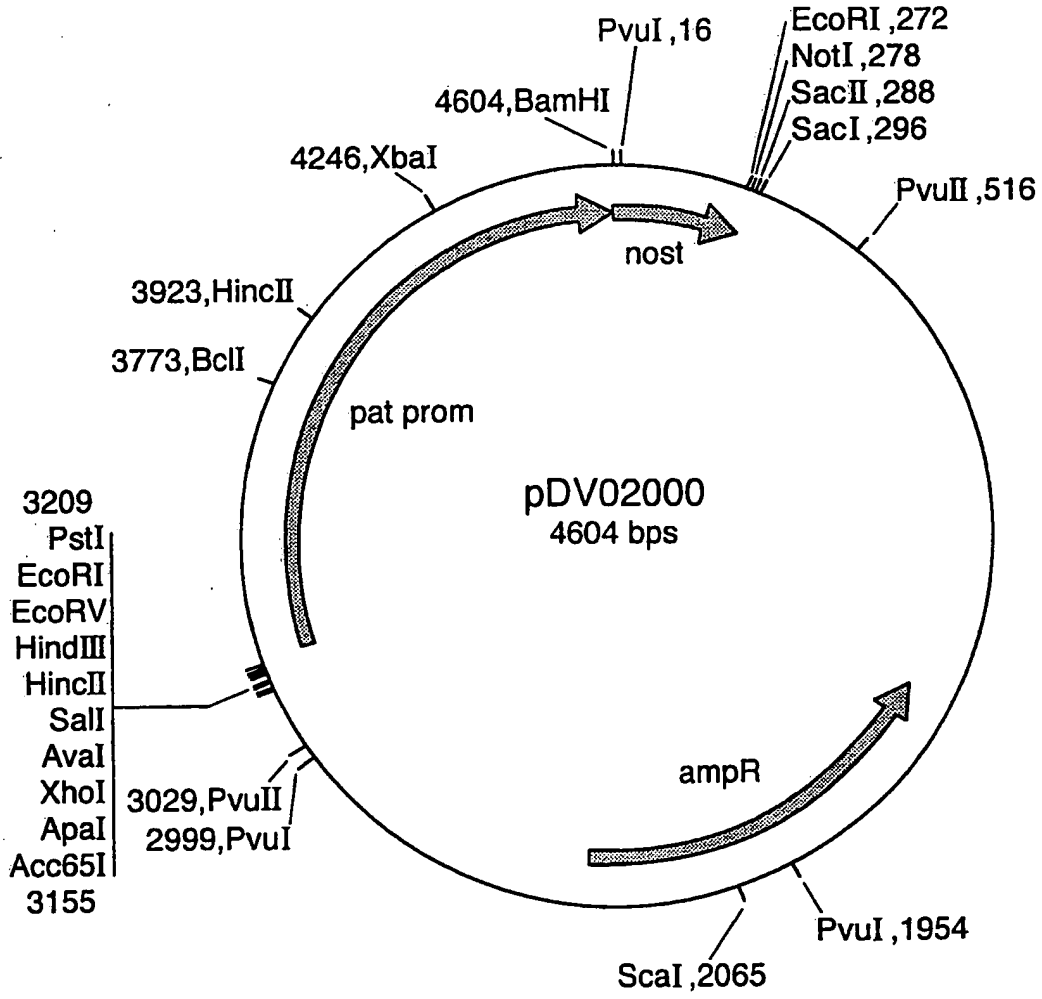
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Fig.6.

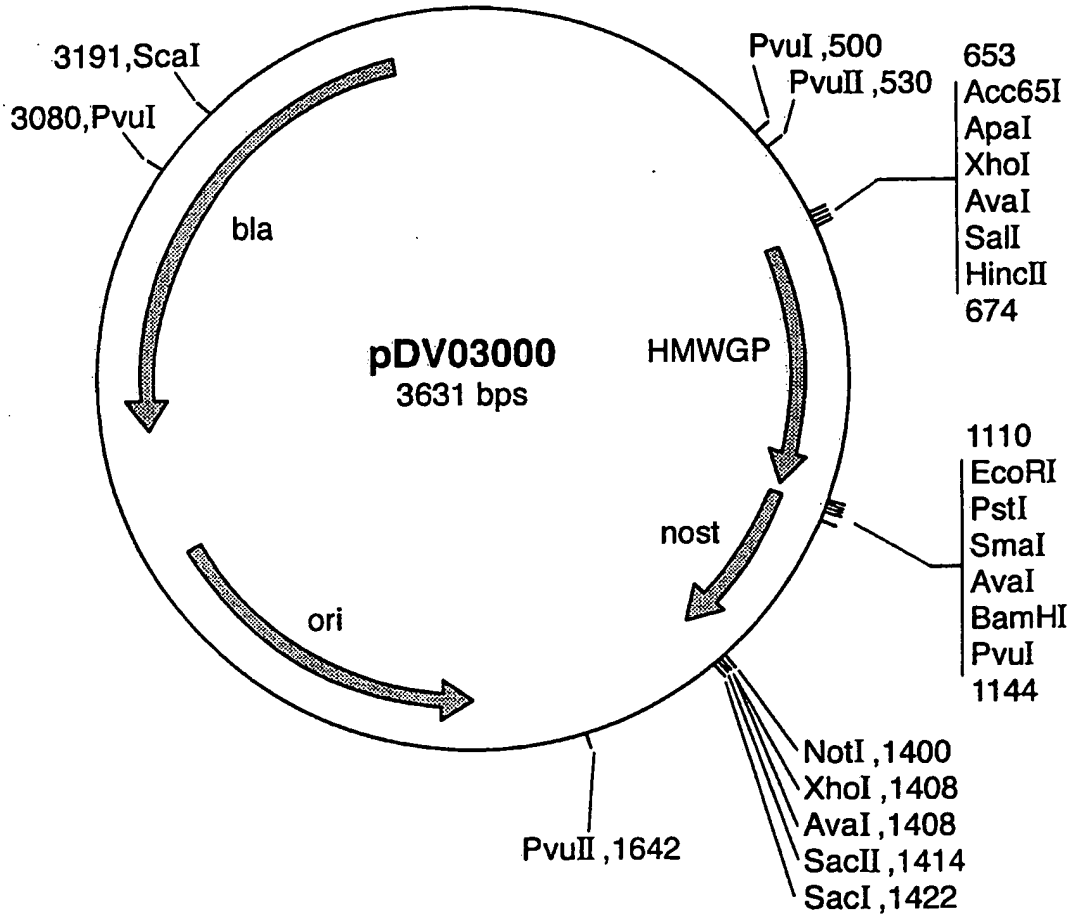
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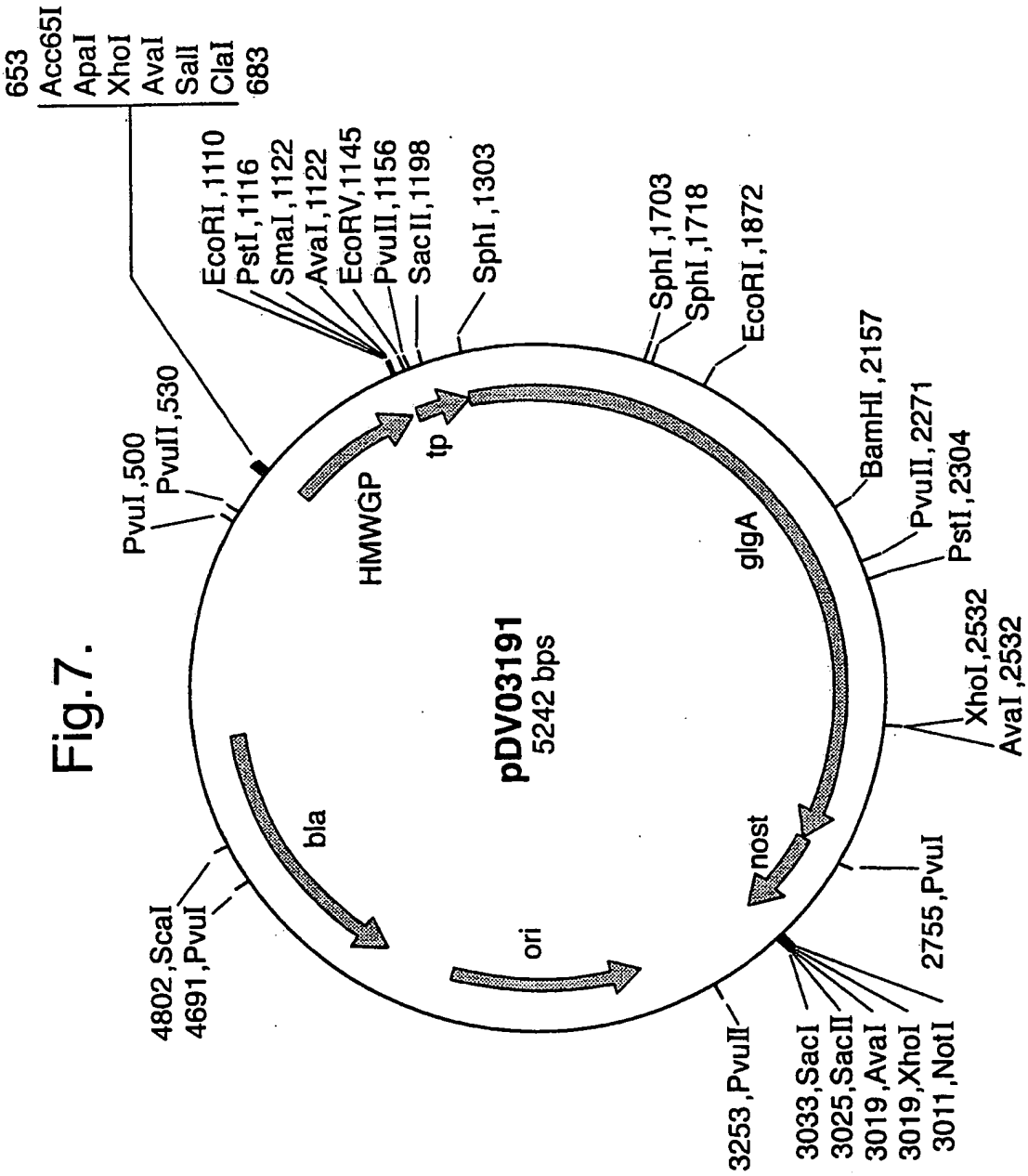
Fig.7.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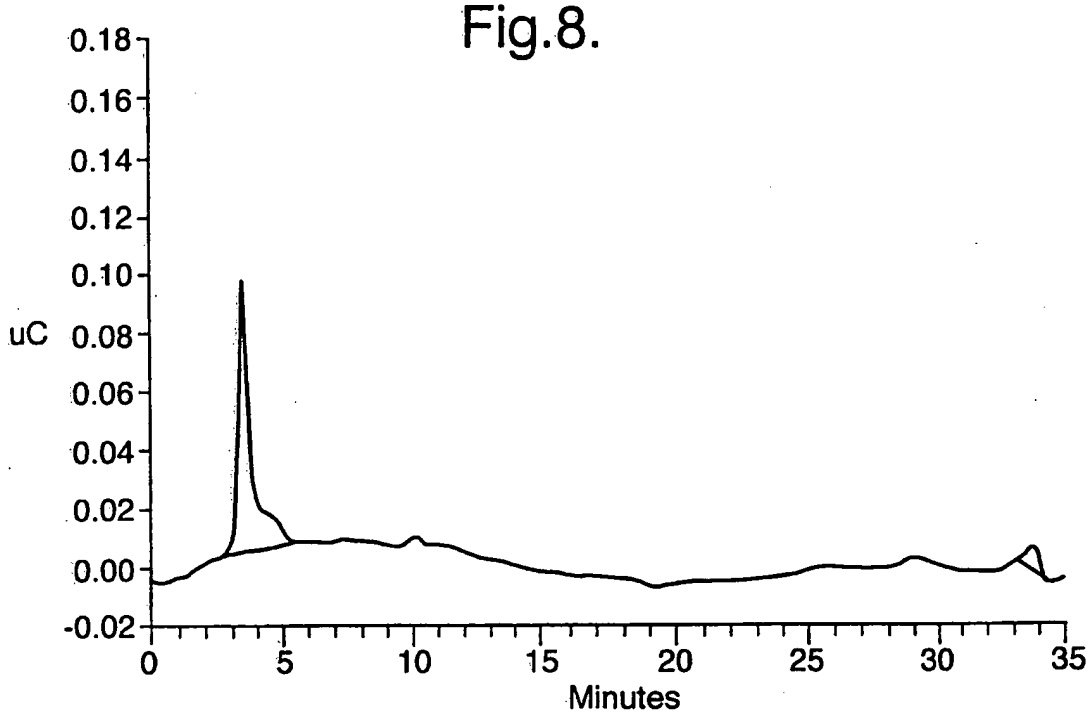
EcoRI,1872
BamHI,2157
PvuII,2271
PstI,2304
XhoI,2532
AvaI,2532
2755,PvuI
3011,NotI
3019,XhoI
3019,AvaI
3025,SacII
3033,SacI
3253,PvuII
4691,PvuI
4802,ScaI
PvuI,500
PvuII,530
HMWGP
tp
glgA
nost
bla
ori
pDV03191
5242 bps

Fig.8.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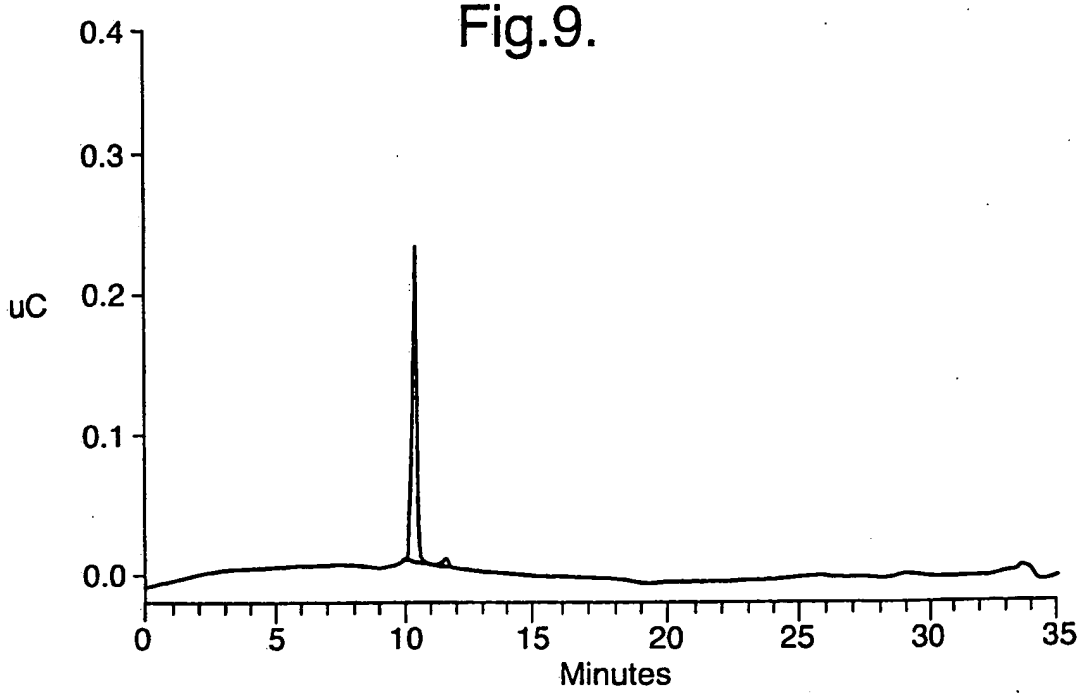
0.16
0.14
0.12
0.10
uC 0.08
0.06
0.04
0.02
0.00
-0.02
0
5
10
15
20
25
30
35
Minutes

Fig.9.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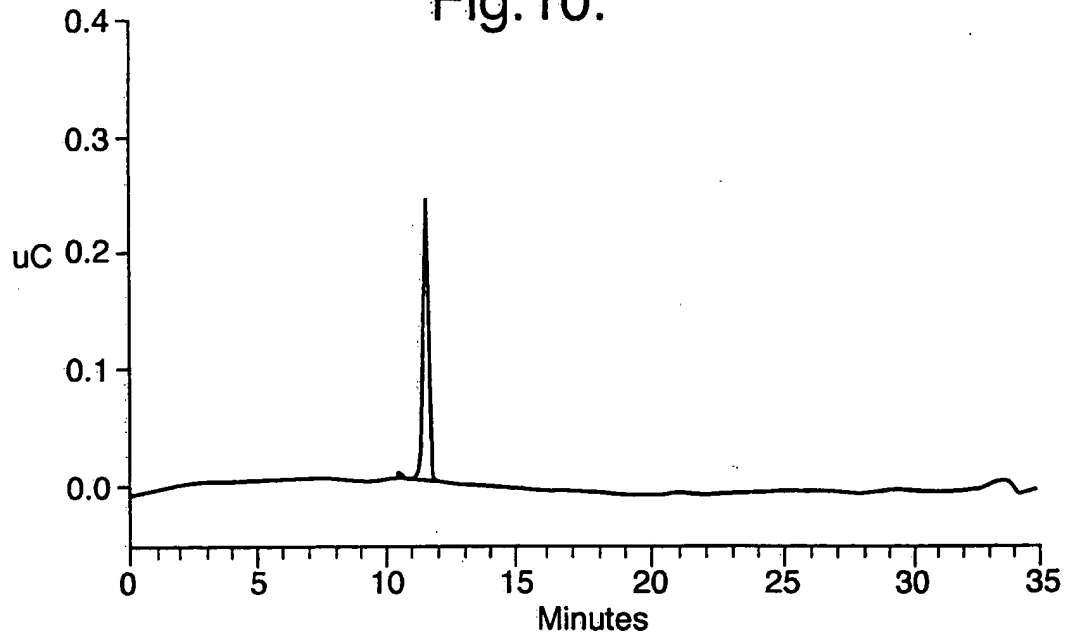
uC 0.2
0.1
0.0
0
5
10
15
20
25
30
35
Minutes

Fig.10.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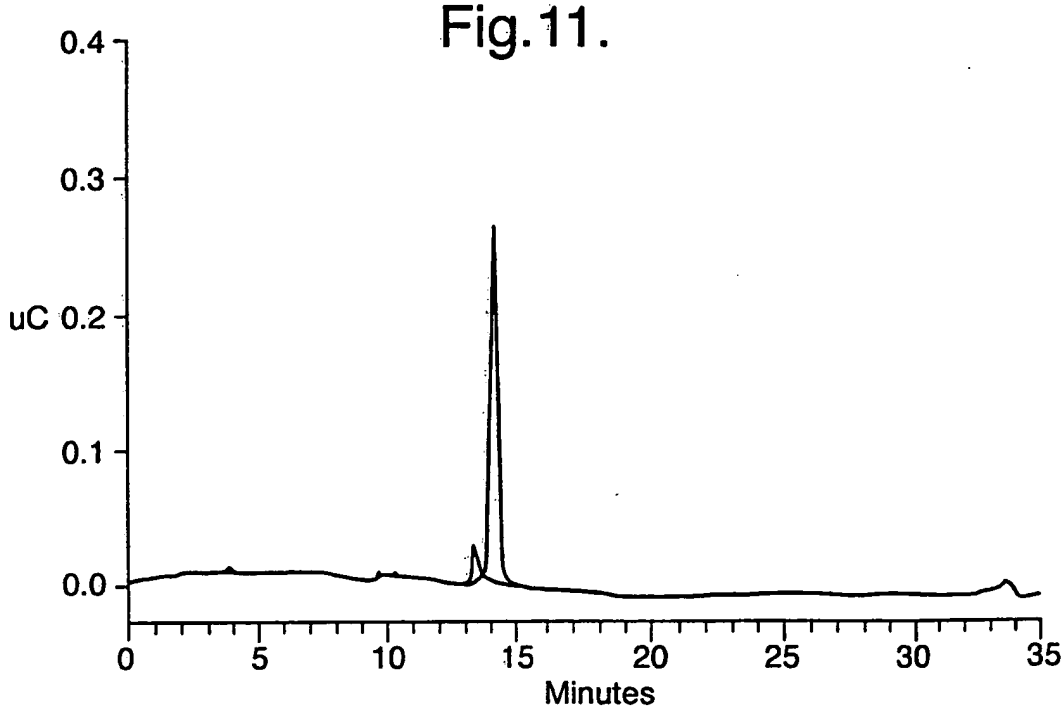
uC 0.2
0.1
0.0
0
5
10
15
20
25
30
35
Minutes

Fig.11.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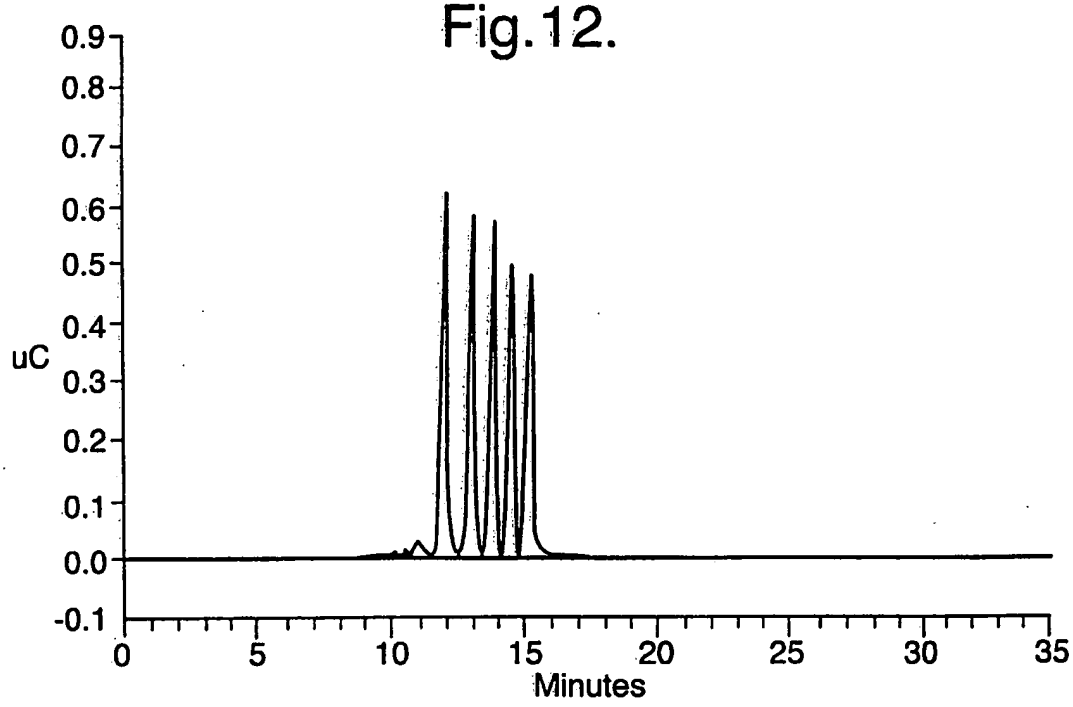
0.3
uC 0.2
0.1
0.0
0
5
10
15
20
25
30
35
Minutes

Fig.12.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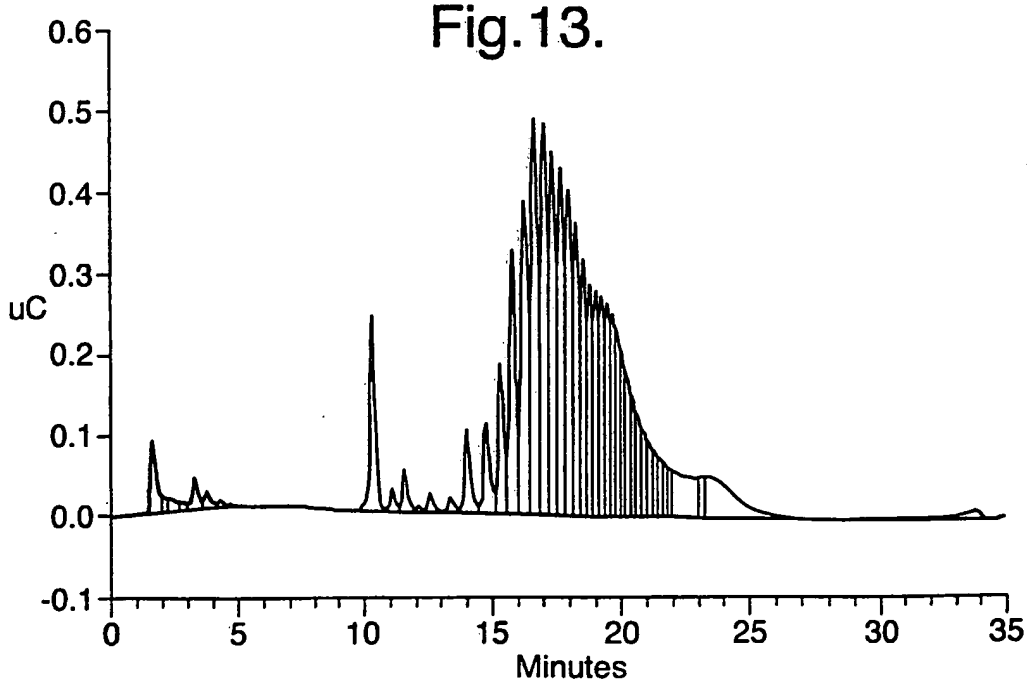
0.3
0.2
0.1
0.0
-0.1
uC
0
5
10
15
20
25
30
35
Minutes

Fig.13.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0.4
0.3
0.2
0.1
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-0.1
uC
0
5
10
15
20
25
30
35
Minutes

Fig.14.

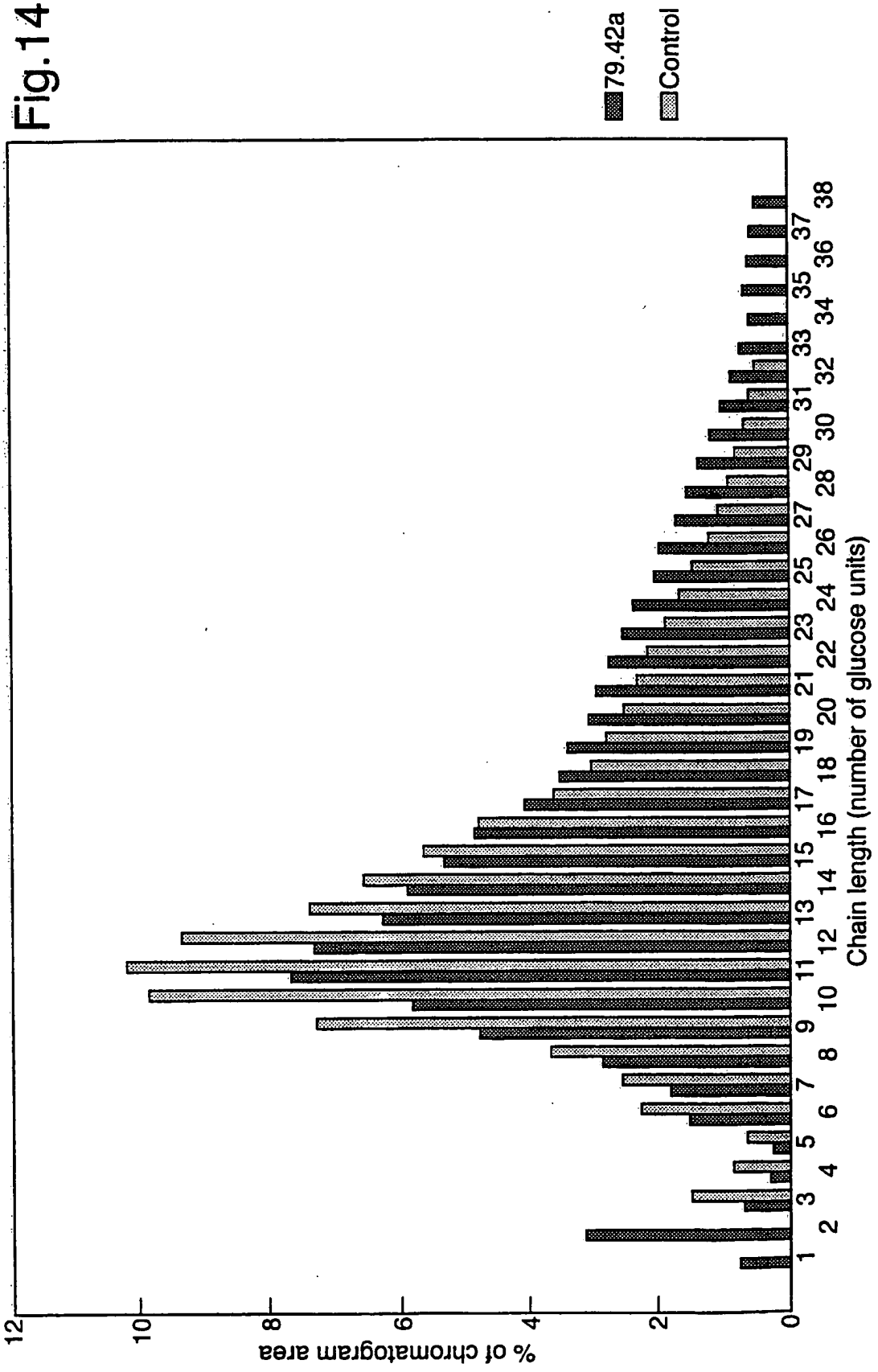

79.42a
Control
% of chromatogram area
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
Chain length (number of glucose units)

Fig.15.

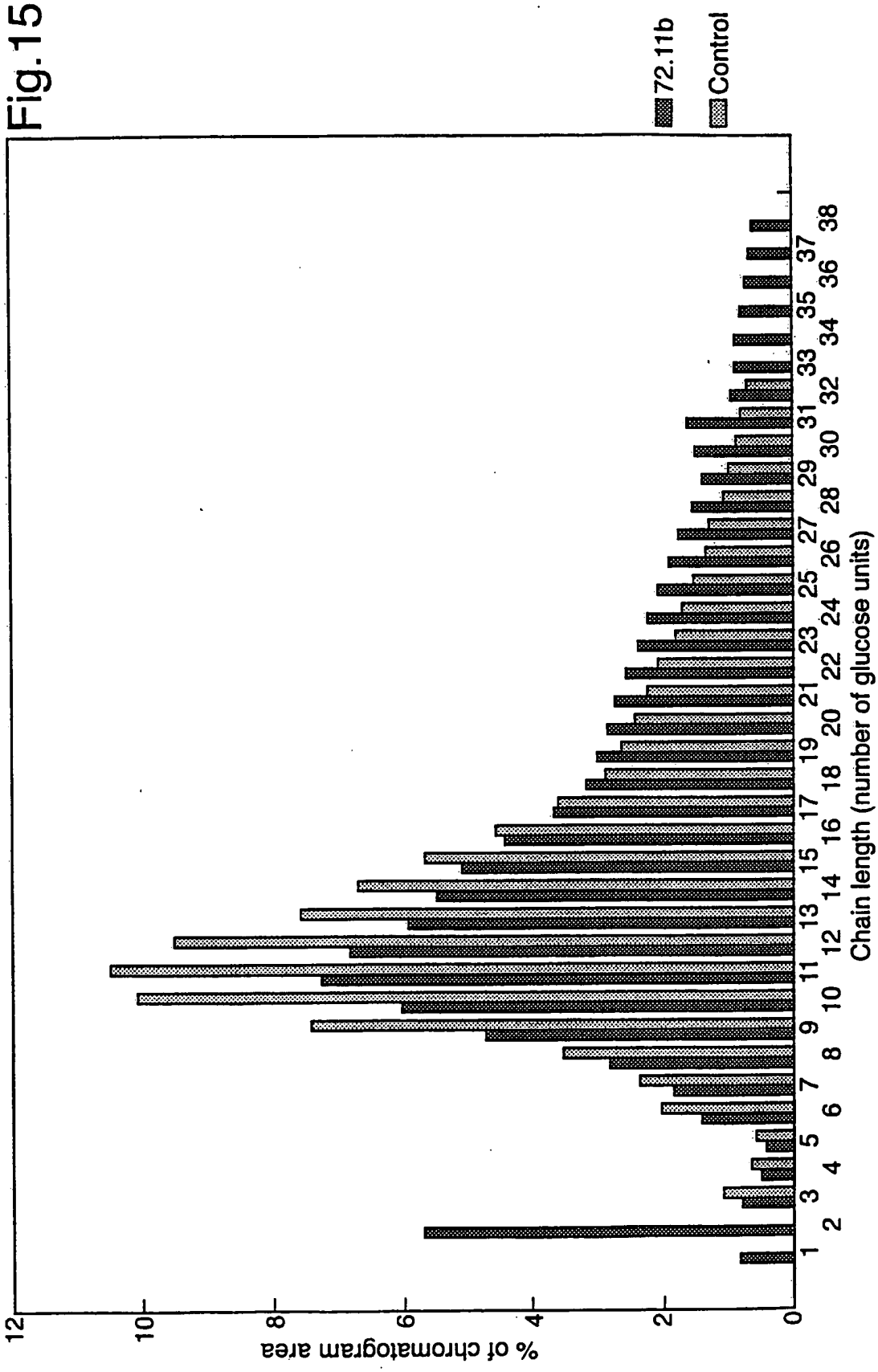

72.11b
Control
% of chromatogram area
0
2
4
6
8
10
12
Chain length (number of glucose units)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

Fig.16.

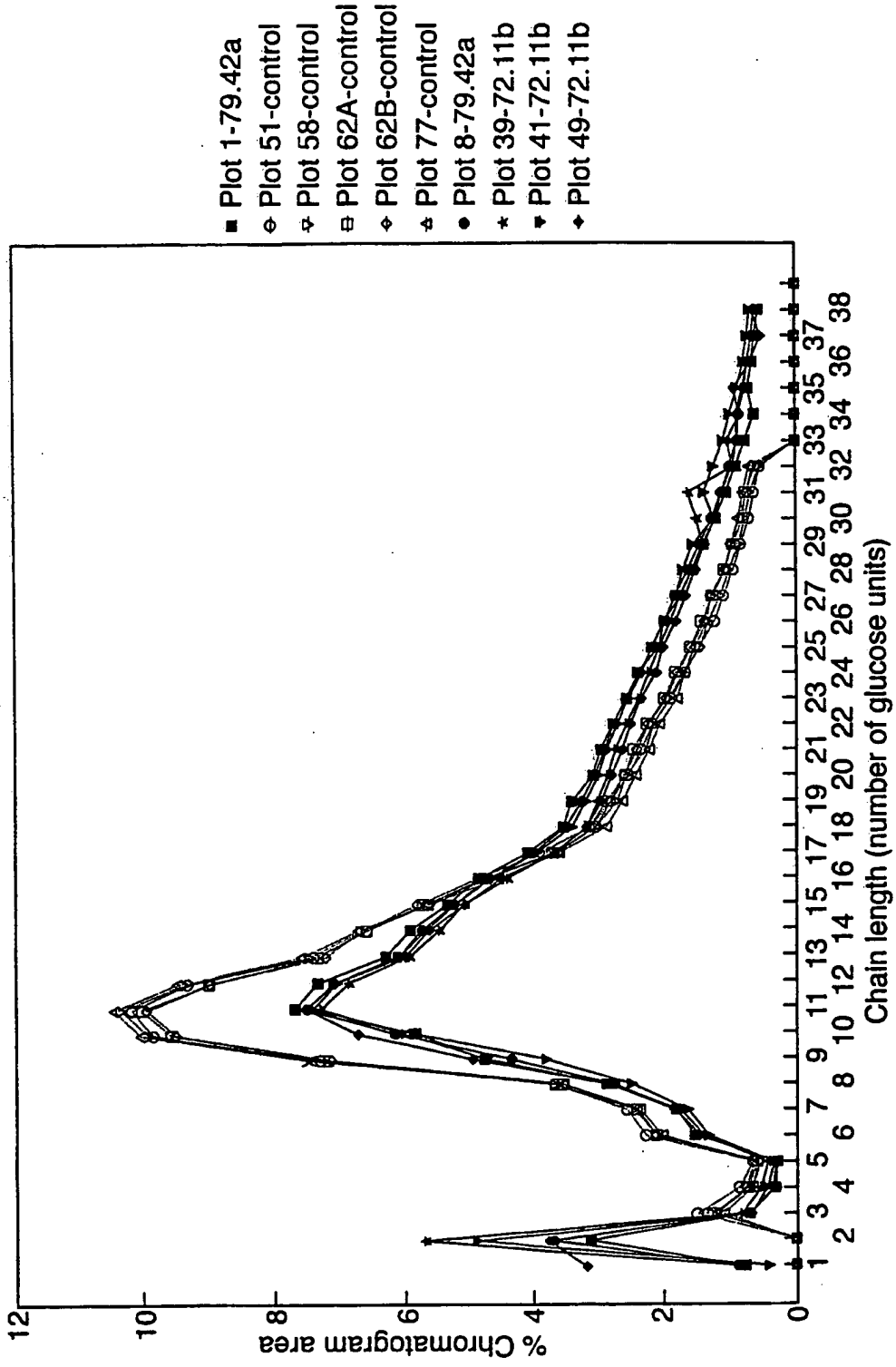
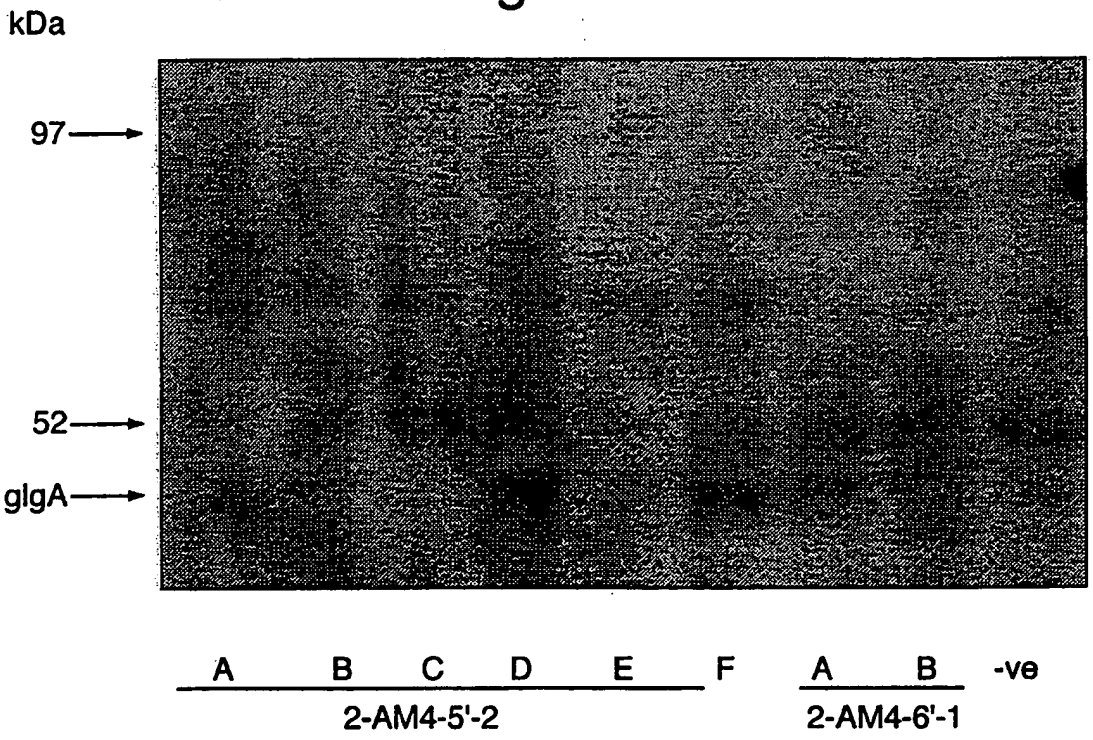

Plot 1-79.42a
Plot 51-control
Plot 58-control
Plot 62A-control
Plot 62B-control
Plot 77-control
Plot 8-79.42a
Plot 39-72.11b
Plot 41-72.11b
Plot 49-72.11b
% Chromatogram area
0
2
4
6
8
10
12
Chain length (number of glucose units)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

Fig.17.



SEQUENCE LISTING

(1) **GENERAL INFORMATION**

(i) **APPLICANTS:**

(A) NAME:	Advanced Technologies (Cambridge) Limited
(B) STREET:	Globe House
(C) CITY:	1 Water Street
(D) STATE:	London
(E) COUNTRY:	England
(F) POSTAL CODE:	WC2R 3LA

(ii) **TITLE OF INVENTION:** Genetically Modified Plants with altered Starch

(iii) **NUMBER OF SEQUENCES:** 9

(iv) **CORRESPONDENCE ADDRESS:**

(A) ADDRESSEE:	British American Tobacco (Investments) Limited
(B) STREET:	Regents Park Road
(C) CITY:	Southampton
(D) STATE:	Hampshire
(E) COUNTRY:	England
(F) POSTAL CODE:	SO15 8TL

(v) **COMPUTER READABLE FORM:**

(A) MEDIUM TYPE:	Diskette 3.50 inch
(B) COMPUTER:	Compaq Deskpro
(C) OPERATING SYSTEM	MS-DOS Windows 95
(D) SOFTWARE:	Microsoft Word 97

(vi) **CURRENT APPLICATION DATA:**

(A) APPLICATION NUMBER:	Not yet known
(B) CLASSIFICATION:	Not yet known

(viii) **ATTORNEY/AGENT INFORMATION:**

(A) NAME:	Mrs. M.R. Walford / Mr.K.J.H. MacLean
(B) REFERENCE:	RD-ATC-20

(ix) **TELECOMMUNICATION INFORMATION:**

(A) TELEPHONE:	01703 777155
(B) TELEFAX:	01703 779856

(2) **INFORMATION FOR SEQ. ID. NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	1467 bps
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** cDNA to mRNA

(vii) **IMMEDIATE SOURCE:**
(B) CLONE: pBS17R

(ix) **FEATURES:**
(A) NAME: CDS ssu transit peptide
(B) LOCATION: 1 to 169

(ix) **FEATURES:**
(A) NAME: CDS *glgC16*
(B) LOCATION: 172 to 146

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO: 1:

```
   1 ATGGCTTCTA TGATATCCTC TTCAGCTGTG ACTACAGTCA GCCGTGCTTC  50
  51 TACGGTGCAA TCGGCCGCGG TGGCTCCATT CGGCGGCCTC AAATCCATGA 100
 101 CTGGATTCCC AGTTAAGAAG GTCAACACTG ACATTACTTC CATTACAAGC 150
 151 AATGGTGGAA GAGTAAAGTG CATGCTTAGT TTAGAGAAGA ACGATCACTT 200
 201 AATGTTGGCG CGCCAGCTGC CATTGAAATC TGTTGCCCTG ATACTGGCGG 250
 251 GAGGACGTGG TACCCGCCTG AAGGATTTAA CCAATAAGCG AGCAAAACCG 300
 301 GCCGTACACT TCGGCGGTAA GTTCCGCATT ATCGACTTTG CGCTGTCTAA 350
 351 CTGCATCAAC TCCGGGATCC GTCGTATGGG CGTGATCACC CAGTACCAGT 400
 401 CCCACACTCT GGTGCAGCAC ATTCAGCGCG GCTGGTCATT CTTCAATGAA 450
 451 GAAATGAACG AGTTTGTCGA TCTGCTGCCA GCACAGCAGA GAATGAAAGG 500
 501 GGAAAACTGG TATCGCGGCA CCGCAGATGC GGTCACCCAA AACCTCGACA 550
 551 TTATCCGTCG TTATAAAGCG GAATACGTGG TGATCCTGGC GGGCGACCAT 600
 601 ATCTACAAGC AAGACTACTC GCGTATGCTT ATCGATCACG TCGAAAAAGG 650
 651 TGTACGTTGT ACCGTTGTTT GTATGCCAGT ACCGATTGAA GAAGCCTCCG 700
 701 CATTTGGCGT TATGGCGGTT GATGAGAACG ATAAAACTAT CGAATTCGTG 750
 751 GAAAAACCTG CTAACCCGCC GTCAATGCCG AACGATCCGA GCAAATCTCT 800
 801 GGCGAGTATG GGTATCTACG TCTTTGACGC CGACTATCTG TATGAACTGC 850
```

```
 851 TGGAAGAAGA CGATCGCGAT GAGAACTCCA GCCACGACTT TGGCAAAGAT 900

 901 TTGATTCCCA AGATCACCGA AGCCGGTCTG GCCTATGCGC ACCCGTTCCC 950

 951 GCTCTCTTGC GTACAATCCG ACCCGGATGC CGAGCCGTAC TGGCGCGATG 1000

1001 TGGGTACGCT GGAAGCTTAC TGGAAAGCGA ACCTCGATCT GGCCTCTGTG 1050

1051 GTGCCGAAAC TGGATATGTA CGATCGCAAT TGGCCAATTC GCACCTACAA 1100

1101 TGAATCATTA CCGCCAGCGA AATTCGTGCA GGATCGCTCC GGTAGCCACG 1150

1151 GGATGACCCT TAACTCACTG GTTTCCGACG GTTGTGTGAT CTCCGGTTCG 1200

1201 GTGGTGGTGC AGTCCGTTCT GTTCTCGCGC GTTCGCGTGA ATTCATTCTG 1250

1251 CAACATTGAT TCCGCCGTAT TGTTACCGGA AGTATGGGTA GGTCGCTCGT 1300

1301 GCCGTCTGCG CCGCTGCGTC ATCGATCGTG CTTGTGTTAT TCCGGAAGGC 1350

1351 ATGGTGATTG GTGAAAACGC AGAGGAAGAT GCACGTCGTT TCTATCGTTC 1400

1401 AGAAGAAGGC ATCGTGCTGG TAACGCGCGA AATGCTACGG AAGTTAGGGC 1450

1451 ATAAACAGGA GCGATAA
```

(2) **INFORMATION FOR SEQ. ID. NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	421 base pairs
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Double stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Genomic DNA

(ix) **FEATURES:**

(A) NAME:	Triticum aestivum
(B) LOCATION:	var CIMMYT

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:2:

```
   1 CCCAGCTTTG AGTGGCCGTA GATTTGCAAA AGCAATGGCT AACAGACACA  50

  51 TATTCTGCCA AACCCCAAGA AGGATAATCA CTTTTCTTAG ATAAAAAAGA 100

 101 ACAGACCAAT ATACAAACAT CCACACTTCT GCAAACAATA CATCAGAACT 150
0
 151 AGGATTACGC CGATTACGTG GCTTTAGCAG ACTGTCCAAA AATCTGTTTT 200

 201 GCAAAGCTCC AATTGCTCCT TGCTTATCCA GCTTCTTTTG TGTTGGCAAA 250
```

```
251 CTGCGCTTTT CCAACCGATT TTGTTCTTCT CGCGCTTTCT TCTTAGCCTA 300

301 AACAAACCTC ACCGTGCACG CAGCCATGGT CCTGAACCTT CACCTCGTCC 350

351 CTATAAAAGC CTAGCCAACC TTCACAATCT TATCATCACC CACAACACCG 400

401 AGCACCACAA ACTAGAGATC C                                421
```

(2) **INFORMATION FOR SEQ. ID. NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	43 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 3' end of ssu transit peptide
(B) LOCATION:	1 to 19
(A) NAME:	Domain complimentary to 5' end of *glgA* CDS
(B) LOCATION:	20 to 43

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:3:

```
1 TGGTGGAAGA GTAAAGTGCA TGCAGGTTTT ACATGTATGT TCA      43
```

(2) **INFORMATION FOR SEQ. ID. NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	54 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 3' end of *glgC* coding sequence
(B) LOCATION:	1 to 19

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 3' end of *glgA* CDS
(B) LOCATION:	26 t 54

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID NO:4:

1 TCGCTCCTGT TTATGCCCTA GATCTCTATT TCGAGCGATA

41 GTAAAGCTCA CGGT

(2) **INFORMATION FOR SEQ. ID. NO:5:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	33 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Complimentary to 5' end of ssu transit peptide
(B) LOCATION:	11 to 33

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:5:

1 ACGTAGATCT ATGGCTTCTA TGATATCCTC TTC 33

(2) **INFORMATION FOR SEQ. ID. NO:6:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	36 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Homologous to 5' end of High Molecular Weight Glutenin Promoter
(B) LOCATION:	10 to 36

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:6:

1 GACATCGATC CCAGCTTTGA GTGGCCGTAG ATTTGC 36

(2) **INFORMATION FOR SEQ. ID. NO:7:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	39 bases

(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single stranded
(D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Complimentary to 3' end of High Molecular Weight Glutenin Promoter
(B) LOCATION: 10 to 39

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:7:

1 GACGAATTCG GATCTCTAGT TTGTGGTGCT CGGTGTTGT 39

(2) **INFORMATION FOR SEQ. ID. NO:8:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 32 bases
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single stranded
(D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Homologous to 5' end of Nopaline synthase terminator
(B) LOCATION: 9 to 32

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:8:

1 CAGGATCCGA ATTTCACCCG ATCGTTCAAA CA 32

(2) **INFORMATION FOR SEQ. ID. NO:9:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 50 bases
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single stranded
(D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Complimentary to 3' end of nopaline synthase terminator
(B) LOCATION: 23 to 50

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:9:

1 GACCGCGGC TCGAGGCGGC CGCCCGATCT AGTAACATAG 40

41 ATGACACCGC 50

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/GB 99/03734

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/82 C12N5/10 C08B30/00 A01H5/00
A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C08B A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 11382 A (CALGENE INC) 9 July 1992 (1992-07-09) cited in the application the whole document	1-23,25
X	SHEWMAKER C K ET AL: "EXPRESSION OF ESCHERICHIA COLI GLYCOGEN SYNTHASE IN THE TUBERS OF TRANSGENIC POTATOES (SOLANUM TUBEROSUM) RESULTS IN A HIGHLY BRANCHED STARCH" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 104, 1 January 1994 (1994-01-01), pages 1159-1166, XP002033871 ISSN: 0032-0889 cited in the application the whole document	1-23,25

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 April 2000	27/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/GB 99/03734

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 09144 A (ZENECA LTD) 28 April 1994 (1994-04-28) cited in the application see the whole document; esp. p.33/34, 51/52, claims	1-23,25
X	WO 94 11520 A (ZENECA LTD ;KEELING PETER LEWIS (GB)) 26 May 1994 (1994-05-26) cited in the application see the whole document; esp. pp.20-24	1-23,25
X	WO 98 44780 A (EXSEED GENETICS, LLC, USA) 15 October 1998 (1998-10-15) see the whole document; esp. pp.23-25, fig. 28,35	1-23,25
A	KORTSTEE A J ET AL: "Expression of Escherichia coli branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin." PLANT JOURNAL, (1996 JUL) 10 (1) 83-90. , XP002135212 the whole document	1-25
A	KORTSTEE A J ET AL: "The influence of an increased degree of branching on the physicochemical properties of starch from genetically modified potato" CARBOHYDRATE POLYMERS, (OCT 1998) VOL. 37, NO. 2, PP. 173-184. PUBLISHER: ELSEVIER SCI LTD, THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD OX5 1GB, OXON, ENGLAND. ISSN: 0144-8617., XP004141125 the whole document	15-19

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/03734

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9211382 A	09-07-1992	US 5349123 A	20-09-1994
		CA 2084079 A	22-06-1992
		EP 0542929 A	26-05-1993
		US 5750875 A	12-05-1998
		US 5969214 A	19-10-1999
WO 9409144 A	28-04-1994	CA 2146998 A	28-04-1994
		AU 690517 B	30-04-1998
		AU 2696492 A	09-05-1994
		EP 0664835 A	02-08-1995
WO 9411520 A	26-05-1994	AU 5428594 A	08-06-1994
WO 9844780 A	15-10-1998	AU 6882898 A	30-10-1998